CHEMOKINES AND CHEMOKINE RECEPTORS IN BRAIN HOMEOSTASIS

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CHEMOKINES AND CHEMOKINE RECEPTORS IN BRAIN HOMEOSTASIS

Topic Editors:
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Virtually involved in all pathologies that present an inflammatory component, it is now evident that, in the central nervous system, chemokines and chemokine receptors possess pleiotropic properties beyond chemotaxis: costitutive brain expression of chemokines and their receptors on endothelial cells, but also on neurons and glia, suggests a role for such molecules in mediating homeostatic cross-talk between cells of the brain perenchyma. Cross-talk between neurons and glia is determinant to the establishment and maintenance of a brain enviroment that ensure normal function, and in particular glial cells are active players that respond to enviromental changes and act for the survival, growth, differentiation and repair of the nervous tissue: in this regard brain endogenous chemokines represent key molecules that play a role in brain development, neurogenesis, neurotransmission and neuroprotection.

As important regulators of peripheral immune response, chemokines are molecules of the immune system that play a central role in coordinating communication between the nervous and the immune systems, in the context of infections and brain injury. Indeed, in phatological processes resulting from infections, brain trauma, ischemia and chronic neurodegenerative diseases, chemokines represent important neuroinflammatory mediators that drive leucocytes trafficking into the central nervous system, facilitating an immune response by targeting cells of the innate and adaptive immune system.

The third edition of the international conference "Chemokines and Chemokine Receptors in the Nervous System", hold in Rome in October 2013, represented an exciting platform to promote discussion among researchers in different disciplines to understand the role of chemokines in brain homoestasis.

This Frontiers Research Topic arises from this conference, and wants to be an opportunity to further discuss and highlight the importance of brain chemokines as key molecules that, not only grant the interplay between the immune and the nervous systems, but in addition drive modulatory functions on brain homeoastasis orchestrating neurons, microglia, and astrocytes communication.

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EDITORIAL
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Editorial Research Topic "Chemokines and chemokine receptors in brain homeostasis"

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Keywords: Chemokines, brain homeostasis, brain pathology, pain, inflammation, neuro-glia cross-talk, glioma stem cell, neurotransmission

The present *Frontiers* eBook "Chemokines and chemokine receptors in brain homeostasis" grew from a delightful conference held in Rome, Italy from 25th to 27th, 2013. It's our hope that this eBook will enable you to sense the conviviality and intellectual ferment of that weekend, as you won't be able to taste the wine or pasta.

The 11 articles in this compilation (Biber and Boddeke, 2014; Clark and Malcangio, 2014; Freitag and Miller, 2014; Guyon, 2014; Hosking and Lane, 2014; Limatola and Ransohoff, 2014; Michlmayr and Lim, 2014; Mony et al., 2014; Rosito et al., 2014; Williams et al., 2014; Würth et al., 2014) comprise a spectrum of chemokine neurobiology much of which will be unfamiliar (and thus, one hopes, fascinating) both to chemokine aficionados and neuroscientists. Only one paper (Mony et al., 2014) addresses purely the best-known aspect of chemokine action in the context of neurological pathology: their role in accumulation of inflammatory blood-derived leukocytes in the central nervous system (CNS). Williams et al. (2014) also study leukocyte recruitment to the CNS but additionally evaluate evidence that CXCL12 (the chemokine on which they focus) can either promote or degrade neural function during altered homeostasis. Limatola and Ransohoff (2014) examine how a neuronal chemokine (CX3CL1) signals to its microglial receptor (CX3CR1) to help determine cell death or survival in the context of varied pathological processes. One group of scientists (Rosito et al., 2014) present their data about how chemokine-mediated cell-cell communication among neurons and glia supports neuronal function after focal cerebral ischemia. Two groups (Hosking and Lane, 2014; Michlmayr and Lim, 2014) integrate these topics (chemokine-regulation of inflammatory host defense; chemokine effects on cell death or survival) by utilizing informative models of encephalitis. Three groups (Biber and Boddeke, 2014; Clark and Malcangio, 2014; Freitag and Miller, 2014) describe their work using chemokine biology to unravel the puzzle of neuropathic pain. There is a heterogeneity of additional topics. Guyon (2014) examines how CXCL12 signaling modulates GABA neurotransmission. Würth et al. (2014) study the same chemokine (CXCL12) now in the guise of an autocrine and paracrine signal to promote growth of glioma stem cells and maintain a supportive microenvironment.

It will be appreciated that the common rubric "Chemokines are chemotactic cytokines" no longer encompasses even a tiny fraction of the activities of these versatile mediators in CNS physiology and pathology. The predominant focus currently lies on CXCL12 and CX3CL1 but other players (ELR+ CXC chemokines; CCL21; CXCL16) also begin to be heard from. Given the pace at which molecular components of development and disease are being identified, it is plausible to hope that this eBook represents only the tip of an iceberg which will calve rapidly into knowledge that promotes the treatment of neurological disorders.

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Chemokine receptor expression by inflammatory T cells in EAE

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Chemokines direct cellular infiltration to tissues, and their receptors and signaling pathways represent targets for therapy in diseases such as multiple sclerosis (MS). The chemokine CCL20 is expressed in choroid plexus, a site of entry of T cells to the central nervous system (CNS). The CCL20 receptor CCR6 has been reported to be selectively expressed by $CD4^+$ T cells that produce the cytokine IL-17 (Th17 cells). Th17 cells and interferon-gamma (IFNγ)-producing Th1 cells are implicated in induction of MS and its animal model experimental autoimmune encephalomyelitis (EAE). We have assessed whether CCR6 identifies specific inflammatory T cell subsets in EAE. Our approach was to induce EAE, and then examine chemokine receptor expression by cytokine-producing T cells sorted from CNS at peak disease. About 7% of CNS-infiltrating $CD4^+$ T cells produced IFNγ in flow cytometric cytokine assays, whereas less than 1% produced IL-17. About 1% of $CD4^+$ T cells produced both cytokines. CCR6 was expressed by Th1, Th1+17 and by Th17 cells, but not by $CD8^+$ T cells. $CD8^+$ T cells expressed CXCR3, which was also expressed by $CD4^+$ T cells, with no correlation to cytokine profile. Messenger RNA for IFNγ, IL-17A, and the Th1 and Th17-associated transcription factors T-bet and RORγt was detected in both $CCR6^+$ and $CXCR3^+$ $CD4^+$ T cells. IFNγ, but not IL-17A mRNA expression was detected in $CD8^+$ T cells in CNS. CCR6 and CD4 were co-localized in spinal cord infiltrates by double immunofluorescence. Consistent with flow cytometry data some but not all $CD4^+$ T cells expressed CCR6 within infiltrates. CD4-negative $CCR6^+$ cells included macrophage/microglial cells. Thus we have for the first time directly studied $CD4^+$ and $CD8^+$ T cells in the CNS of mice with peak EAE, and determined IFNγ and IL17 expression by cells expressing CCR6 and CXCR3. We show that neither CCR6 or CXCR3 align with CD4 T cell subsets, and Th1 or mixed Th1+17 predominate in EAE.

Keywords: mouse, EAE, T cell, chemokine receptor, cytokine

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) whose pathogenesis involves infiltrating immune cells, including T cells. $CD4^+$ T cells play a central role in orchestrating immune responses by secreting cytokines that regulate various cellular functions. Effector $CD4^+$ T cells of Th1 and Th17 subsets are found in MS lesion and can mediate experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Expression of Th1 and Th17 cytokines, IFNγ and IL-17 is detected in MS lesions (Steinman, 2008). EAE can be induced by the adoptive transfer of CNS antigen reactive Th1 cells (Pettinelli and Mcfarlin, 1981; Ando et al., 1989; Merrill et al., 1992; Baron et al., 1993) and Th17 cells (Langrish et al., 2005; Jäger et al., 2009; Domingues et al., 2010). While EAE induced by adoptive transfer of Th1 cells is characterized by infiltrates predominantly comprising of macrophages, EAE induced by Th17 cells is characterized by neutrophil recruitment (Kroenke et al., 2008).

MHC-I restricted $CD8^+$ T cells are also suggested to play pathogenic roles in MS and its different animal models (Huseby et al., 2012). $CD8^+$ T cells are present in the immune infiltrates in MS lesions (Traugott et al., 1983). $CD8^+$ T cells in MS lesions are oligoclonally expanded (Babbe et al., 2000) and outnumber $CD4^+$ T cells as the most frequent T cell subset in MS lesions (Hauser et al., 1986; Babbe et al., 2000). MHC-I molecules that present antigens to $CD8^+$ T cells are highly expressed in astrocytes, oligodendrocytes, and neurons (axons) within the MS lesions suggesting that $CD8^+$ T cells can directly engage these cells (Höftberger et al., 2004).

Migration of activated T cells into the CNS is directed by chemokines (Holman et al., 2011) and mediated by adhesion molecules (Engelhardt and Ransohoff, 2012). Constitutive expression of the chemokine CCL20 in choroid plexus is proposed to act as a gateway for T cells into uninflamed CNS (Axtell and Steinman, 2009; Reboldi et al., 2009). Th17 cells can preferentially express CCR6, the chemokine receptor for CCL20, *in vitro* (Hirota et al., 2007; Pötzl et al., 2008; Singh et al., 2008; Yamazaki et al.,

2008; Reboldi et al., 2009). Based on the preferential expression of CCR6 in Th17, constitutive expression of CCL20 in choroid plexus and the requirement of CCR6 expression in $CD4^+$ T cells for EAE, it is proposed that CCR6 plays a critical role in the entry of Th17 cells into the CNS in EAE and in induction of disease (Reboldi et al., 2009). The chemokine receptor CXCR3 binds CXC chemokines such as CXCL10 and is also of interest in EAE, although consensus is lacking on its precise role (Liu et al., 2005; Muller et al., 2010).

Forced expression of RORγt, the transcription factor critical for Th17 differentiation, can result in CCR6 expression (Ivanov et al., 2006; Hirota et al., 2007). However, RORγt expression in $CD4^+$ T cells does not guarantee CCR6 expression *in vivo*. Although CCR6 expression correlates well with RORγt expressing IL-17 producers, $CD4^+$ T cells that do not produce IL-17 can also express CCR6 (Wang et al., 2009). It is not known whether Th1 cells *in vivo* can also express CCR6.

We have assessed whether CCR6 identifies specific inflammatory T cell subsets in the CNS of mice with EAE, by direct analysis of CNS-infiltrating cells, with minimal manipulation. We find that Th1 outnumber Th17 $CD4^+$ T cells, and that CCR6 is expressed by both, as well as by Th1+17. We also show that $CD8^+$ T cells express CXCR3 rather than CCR6, and do not express IL-17. Thus chemokine receptors do not align with cytokine profiles amongst CNS-infiltrating T cells.

MATERIALS AND METHODS

ANIMALS

C57BL/6 (B6) female mice were purchased from Taconic (Ry, Denmark). Mice were provided with food and water *ad libitum*. The mice were allowed to acclimatize with the environment in animal facility for a week before immunization. The experiments were carried out in accordance with rules and regulations laid down by Danish Justice Ministry Committee on Animal Research (Approval Number 2012-15-2934-00110).

EAE

Mice were immunized by subcutaneous injection of 100 μl emulsion (50 μl on each side) containing myelin oligodendrocyte glycoprotein (MOG) p35-55 (100 μg) and complete Freund's adjuvant (CFA) with heat inactivated *Mycobacterium tuberculosis* H37RA (200 μg; Difco Laboratories, Detroit) in the inguinal region. Animals received an intraperitoneal injection (200 μl) of pertussis toxin (0.3 μg; Sigma-Aldrich, Brøndby, Denmark) at the time of immunization and 2 days post-immunization (dpi). MOG p35-55 was synthesized at the Center for Experimental Bioinformatics (CEBI), Department of Biochemistry and Molecular Biology, University of Southern Denmark.

Mice were monitored for loss of body weight and symptoms associated with EAE. Severity of symptoms were used to grade EAE as follows: Grade 0, asymptomatic; Grade 1, weak or hooked tail; Grade 2, floppy tail indicating complete loss of tonus in tail; Grade 3, floppy tail and hind limb paresis (splaying of limbs, slow or unsteady gait, hind limbs slip off the bars while walking on the lids of the cages), Grade 4: floppy tail and unilateral hind limb paralysis; Grade 5, floppy tail and bilateral hind limb paralysis. Animals were killed as the disease peaked, determined by stabilization of the grade for 2 or more days, or when they attained the ethically permitted limit of grade 5. Mice were deeply anaesthetized and perfused intracardially with ice-cold Phosphate Buffered Saline (PBS), and spinal cords were dissected out.

FLOW CYTOMETRY

Spinal cords were collected in ice cold Hanks Balanced Salt Solution (HBSS) (Invitrogen A/S, Taastrup, Denmark). Cell suspensions were prepared by mechanical dissociation and forcing through a 70 mm cell strainer (BD Biosciences, Brøndby, Denmark). Myelin in the samples was removed following centrifugation on 37% isotonic Percoll (GE Healthcare Bio-sciences AB, Uppsala, Sweden).

T cells were stimulated for 9 h in 96 well plates coated with anti-mouse CD3ϵ (clone 145-2C11) in the presence of 1 μl/ml GolgiPlug (BD Biosciences) that was added 2 h after plating, to trap the cytokines within the cells.

The cells were washed and stained with PerCP/Cy5-CD8 (clone 53–6.7), FITC-CD4 (clone GK1.5) or V500-CD4 (clone RM4-5) (BD Biosciences, Brøndby, Denmark), APC- or PE-CCR6 (clone 29-2L17) (Biolegend), PE-IL-17 (clone TC11-18H10.1) (Biolegend), PE-Cy7-IFNγ (clone XMG1.2) (Biolegend) and biotinylated CXCR3 (clone CXCR3-183) (Biolegend) detected using APC- or PE-streptavidin. Individual isotype controls were performed for each sample. Data was collected on LSRII (BD Biosciences, San Jose, CA) and analyzed using FACS DIVA (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

For sorting CCR6 expressing T cells in the CNS, cells were stained with PerCP/Cy5-CD8 (clone 53–6.7), FITC-CD4 (clone GK1.5) and PE-CCR6 (clone 29-2L17). Cells were sorted on a FACSVantage/Diva cell sorter (BD Biosciences) from pooled batches of CNS isolates from 5 mice with MOG p35-55-induced EAE. The experiment was repeated twice to generate three replicate samples of T cells isolated from CNS, from separate EAE inductions.

DOUBLE IMMUNOHISTOCHEMISTRY

Spinal cords were dissected out from PBS perfused mice, placed in 4% paraformaldehyde (PFA, Sigma-Aldrich, Denmark) in PBS, then immersed in 30% Sucrose and frozen as described previously (Mony et al., 2014). Spinal cord sections (16 μm thick) were cut on a cryostat and stored at −80°C. In brief, sections were postfixed in 4% PFA, and after several washes in PBS and PBS containing 0.2% Triton-X100 (PBST), they were then incubated with blocking solution containing 3% Bovine serum albumin in PBST. Sections were stained with PE-CCR6 (clone 29-2L17, Biolegend) and FITC-CD4 (clone GK1.5). Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen-Molecular Probes). Isotype-matched primary antibodies were used to control for non-specific staining. Images for CCR6 expression in $CD4^+$ T cells were acquired using an Olympus BX51 microscope (Olympus, Denmark) connected to an Olympus DP71 digital camera, and combined using Adobe Photoshop CS version 8.0 to visualize double-labeled cells.

QUANTITATIVE REAL-TIME PCR

RNA was extracted from sorted cells according to the manufacturer's protocol for TRIzol (Invitrogen Life Technologies). Moloney murine leukemia virus RT (Invitrogen Life Technologies) was used to synthesize cDNA from the total RNA using random hexamer primers. Quantitative Real-Time Reverse Transcriptase- PCR assays (qRT-PCR) for IFN-γ, IL-17, T-bet, ROR-γt, and 18S rRNA (Applied Biosystems) were performed using ABI Prism 7300 Sequence Detection Systems (Applied Biosystems, Foster City, CA). The following primer and probe sequences were used: IFN-γ (Forward CATTGAAAGCCTAGA AAGTCTGAATAAC, Reverse TGGCTCTGCAGGATTTTCATG, Probe TCACCATCCTTTTGCCAGTTCCTCCAGMGB), IL-17 (Forward CTCCAGAAGGCCCTCAGACTAC, Reverse TGTGGT GGTCCAGCTTTCC, Probe ACTCTCCACCGCAATGAMGB), ROR-γt (Forward CCGCTGAGAGGGCTTCAC, Reverse TGCA GGAGTAGGCCACATTACA, Probe AAGGGCTTCTTCCGCC GCAGCCAGCAG TAMRA). The expression of T-bet and GM-CSF was determined using Mm01299452-g1- and Mm00438328-m1 TaqMan gene expression assays (Applied Biosystem), respectively. Relative RNA levels in the samples were determined using standard curves prepared from four-fold serial dilutions of cDNA from a reference sample. Relative expression levels of genes were normalized to 18S rRNA in the samples.

STATISTICAL ANALYSIS

Data was analyzed using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, California, USA). CCR6 expression was analyzed using nonparametric Mann-Whitney *t*-test. CCR6 expression in IFNγ, IL-17 and IFNγ+IL-17+ CD4+ T cells was analyzed by one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

RESULTS

EAE was induced by immunization with MOGp35-55, a commonly used encephalitogen (Gold et al., 2006). Onset of disease was usually at about day 10 and progression showed a rapid increase in clinical score that levelled off after a few days. Our definition of peak disease was two sequential days where clinical score did not increase, at which point mice were sacrificed for molecular and histological analyses. **Figure 1A** shows the disease course for the animals in this study.

Infiltrating lymphocytes and leukocytes were analyzed by flow cytometry. Gating strategies are shown in **Figure 1B**. Characteristically for EAE, populations were quite heterogeneous, including TCR β^+ T cells and $CD11b^+$ myeloid cells (macrophages, neutrophils and dendritic cells) (Zehntner et al., 2005; Gold et al., 2006; Toft-Hansen et al., 2011). The majority (78.9 ± 2.3%, $n = 10$) of T cells were $CD4^+$. Expression of CCR6 by $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry. Whereas a large proportion (15.9 ± 7.5%, $n = 23$) of $CD4^+$ T cells expressed CCR6, almost no $CD8^+$ T cells expressed this receptor (**Figure 1B**). We have described elsewhere that the majority of $CD8^+$ T cells expressed CXCR3, which was variably expressed by $CD4^+$ T cells (Mony et al., 2014). We did not directly assess whether individual T cells expressed both chemokine receptors.

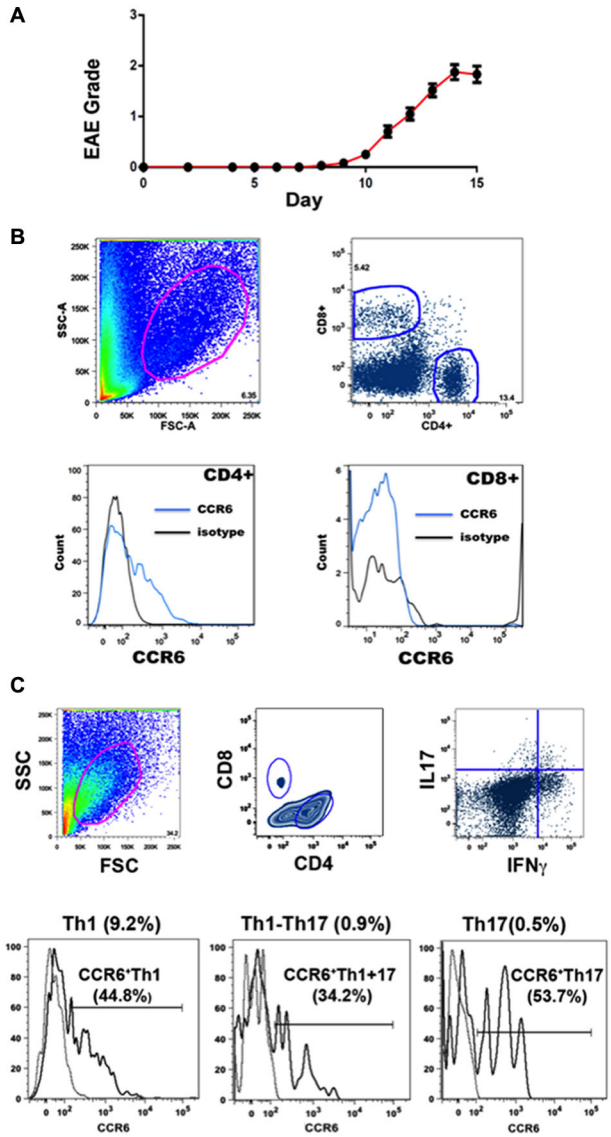

FIGURE 1 | EAE and flow cytometry. (A) Progression of EAE in mice immunized with MOGp35-55 (Mean clinical scores ± SEM, $n = 184$). **(B)** Gating strategies for flow cytometry analysis. CCR6 expression was detected in $CD4^+$ T cells but not $CD8^+$ T cells in CNS. Cells in the spinal cord were gated on the basis of size and granularity (top left), followed by CD4 and CD8 expression (top right) and CCR6 expression ($CD4^+$, lower left) and ($CD8^+$, lower right). Each sample was individually controlled for binding of isotype-matched antibody of irrelevant specificity. **(C)** Gating strategy for cell sorting. CCR6 expression was detected in both IL17 and IFNγ producing $CD4^+$ T cells in spinal cord. Representative gating scheme depicting the morphological gate (top left), CD4 and CD8 gates (top middle), and IFNγ vs IL17 expression in $CD4^+$ T cells. Subsequently, IFNγ, IL17 and IFNγ-IL17 dual producers were gated reative to isotype control for CCR6 expression (bottom histograms). Proportions of each subset within CNS $CD4^+$ isolates are shown on top of each profile, and the percentages that expressed CCR6 are shown with the histograms.

Expression of inflammatory cytokines was measured by flow cytometric intracellular cytokine staining. Th1 IFNγ-producing $CD4^+$ T cells (6.8 ± 0.7%, $n = 8$) greatly outnumbered other

subsets, and together with those that produced both IFNγ and IL-17 (Th1+17) (0.9 ± 0.2%, n = 8), cells producing IFNγ constituted over 90% of cytokine-producing CD4$^+$ T cells in the CNS (**Figure 1C**) (also see Mony et al., 2014). Th17 IL-17-producing CD4$^+$ T cells constituted 0.7 ± 0.1% (n = 8) of the total. CD8$^+$ T cells produced IFNγ but did not produce IL-17 to any significant extent (Mony et al., 2014). Notably, CCR6 was expressed by 30–60% of CD4$^+$ T cells in intracellular cytokine assays, regardless of their cytokine profiles (**Figure 1C**). There was no significant bias towards or against CCR6 expression by Th1, Th17 or Th1+17 subsets (**Figures 1C**, **2**).

Expression of cytokines and of transcription factors that control expression of key cytokines was also examined byQRT-PCR analysis of cDNA from CD4$^+$ T cell populations that were sorted on the basis of CCR6 and CXCR3 expression from CNS infiltrates of mice with peak EAE. **Figure 3** shows that, as for intracellular cytokines, there was no significant bias towards or against expression of IFNγ or IL-17 message on the basis of surface expression of either of these chemokine receptors. This was also true for GM-CSF, a cytokine that has been implicated as a direct encephalitogenic mediator in EAE (Kroenke et al., 2010; Codarri et al., 2011). Similarly, no bias was seen for expression of Tbet and RORγt, the transcription factors that control expression of IFNγ and IL-17, respectively. Lack of detectable signal in some of the sorted populations of CD4$^+$ CCR6$^+$ T cells likely reflects low amounts of RNA in those samples. Populations sorted on the basis of lack of expression of either CCR6 or CXCR3 showed equivalent if not greater levels of message for all cytokines and transcription factors as those sorted for chemokine receptor expression, although populations identified on the basis of lack of expression of a single receptor are intrinsically less informative. CD8$^+$ T cells sorted from the CNS of mice with peak EAE contained equivalent levels of mRNA for both IFNγ and GM-CSF to those in CD4$^+$ cells, but did not express detectable IL-17 message (**Figure 3**).

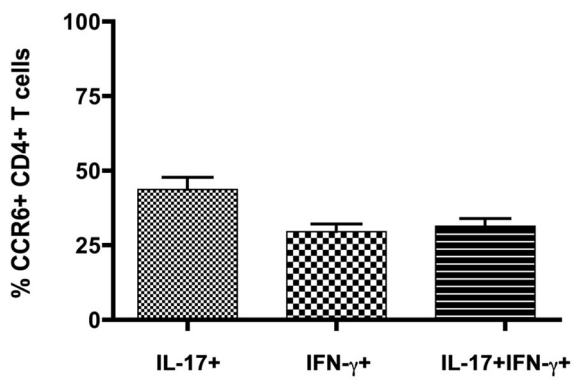

FIGURE 2 | CCR6 expression by IFNγ- and IL17-producing CD4$^+$ T cells in the CNS. Cytokine producing T cells isolated from CNS of mice with severe EAE were detected by flow cytometry following *in vitro* restimulation and intracellular cytokine staining. CCR6 expression was analyzed on cells gated for CD4 and intracellular IL-17 and IFNγ, relative to isotype control. Bars show means ± SEM (n = 8).

We then localized CCR6-expressing cells within infiltrates by immunofluorescence microscopy. **Figure 4** shows that CCR6$^+$ cells were numerous within infiltrates in spinal cord of mice with peak disease, and that many of them co-expressed CD4. These are included within the CD4$^+$ CCR6$^+$ cells that were sorted and analyzed by flow cytometry. There were also a significant number of CD4$^+$ cells that did not express CCR6, which may be assumed to include CXCR3$^+$ CD4$^+$ T cells. CCR6$^+$ cells that did not express CD4 were also observed (arrows). Staining with antibody against GFAP and morphology excluded that these were astrocytes (not shown). For technical reasons it was difficult to co-localize CCR6 with myeloid markers in tissue sections, so we used flow cytometry to determine whether CD11b$^+$ cells also expressed CCR6. Those data are shown in **Figure 4B**. In two separate analyses we could show an increased proportion of CCR6-expressing CD11b$^+$CD45^{high} (eg infiltrating, blood-derived) cells. Almost no CD11b$^+$ CD45^{dim} microglia from the same isolates could be shown to express CCR6, although we cannot exclude that a few of these cells were CCR6$^+$—neither of these populations were further examined or localized. As expected, there was a significant proportion of cells expressing CCR6 within the CD45^{high} CD11b-negative population, which include infiltrating T cells. Flow cytometry confirmed that CD8$^+$ cells in CNS did not express CCR6 (not shown).

Thus, both CD4$^+$ T cells and macrophages expressed the CCR6 chemokine receptor in spinal cord infiltrates of mice with EAE.

DISCUSSION

Interplay between CNS- and immune-derived signals is central to induction and regulation of neuroinflammatory diseases such as MS. The possibility that chemokines might selectively recruit T cells with distinct functional capability opens scenarios that are both of fundamental interest as well as offering therapeutic options. We have asked whether T cells that were recruited to the CNS of mice with EAE show selective expression of the CCR6 chemokine receptor, that had been identified as aligning with the IL-17-producing CD4$^+$ Th17 cytokine subset in studies of experimentally polarized T cells. A previous study had addressed this by taking a post-hoc approach of measuring Th subsets that had already infiltrated to induce severe EAE, and determining their chemokine receptor expression, but had not examined CCR6 or Th17 within CNS infiltrates (Fife et al., 2001). Taking a similar approach we demonstrate, as far as we know for the first time, that the Th1 and Th1+17 subsets, both producing IFNγ, overwhelmingly predominated in CNS, and that many Th1 as well as Th1+17 expressed CCR6. We also find that almost no CD8$^+$ T cells in CNS expressed CCR6 or IL-17, but were overwhelmingly IFNγ-producers that expressed CXCR3. Both CD4$^+$ and CD8$^+$ T cells expressed GM-CSF, and expression of the Th1 and Th17-associated transcription factors T-bet and RORγt aligned with IFNγ and IL-17 respectively.

These findings support three broad interpretative conclusions: (1) CCR6 can be expressed by Th1 and by Th1+17 as well as by Th17 in CNS; (2) IFNγ-producing T cells are a major component of the neuroinflammatory response in EAE; and (3) The spectrum of chemokines and their receptors that control immune

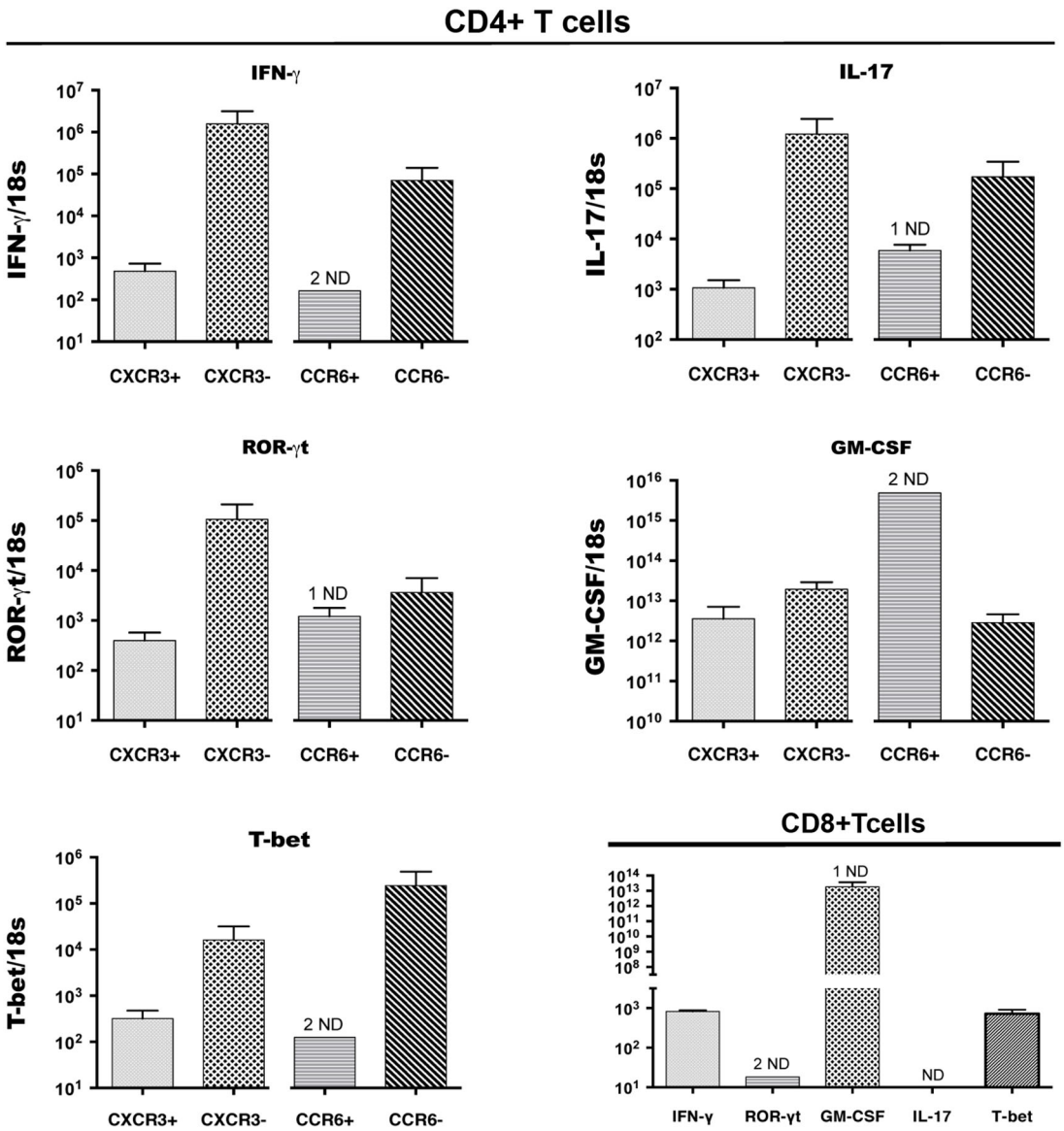

FIGURE 3 | Cytokine and transcription factor (TF) gene expression by CCR6$^+$CD4$^+$, CXCR3$^+$CD4$^+$ and CD8$^+$ T cells in the CNS. CD4$^+$ T cells expressing CXCR3 or CCR6, and CD8$^+$ T cells, were sorted from spinal cords of mice with EAE, pooled from groups of 5 mice, in three different experiments. IFNγ, IL-17, GM-CSF, RORγt, T-bet mRNA expression was detected byQRT-PCR in CXCR3-expressing and CCR6-expressing, as well as receptor-negative CD4$^+$ T cells from the same sorts (Top 4 and bottom left panels). Bottom right panel: CD8$^+$ T cells in the CNS expressed IFNγ, GM-CSF, RORγt, Tbet mRNA, but not IL-17. The y axis shows relative levels of expression (compared to a standard curve) as a ratio to 18S rRNA levels in the same sample. ND: not detectable (1 indicates 1 sample only ND).

infiltration to the CNS is likely to be quite broad. These will be discussed in turn.

CCR6 is the chemokine receptor for CCL20 (liver activation regulated chemokine, LARC or macrophage inflammatory protein-3α, MIP3α) (Baba et al., 1997; Greaves et al., 1997; Hieshima et al., 1997; Rossi and Zlotnik, 2000). The constitutive expression of CCL20 in choroid plexus is proposed to act as a gateway for T cells into uninflamed CNS (Axtell and Steinman, 2009; Reboldi et al., 2009). CCL20 and CCR6 expression are upregulated in the spinal cord in EAE (Serafini et al., 2000). CCL20 is expressed mainly by leukocytes infiltrating the CNS of SJL mice at the onset (acute phase) of relapsing-remitting EAE. CCL20 is also expressed in astrocytes after disease relapses (chronic phase) in the SJL/J EAE model (Serafini et al., 2000; Ambrosini et al., 2003). The cytokines IL1β, IL6, TNFα and combinations of IL1β and TNFα, IL6 and IL-17 can induce CCL20 in astrocyte cultures, whereas IFNγ and IL-17 do not (Ambrosini et al., 2003; Kang et al., 2010; Meares et al., 2012). IL1β, IL6 and TNFα expression are elevated in the brains of mice before the onset of symptoms in EAE (Murphy et al.,

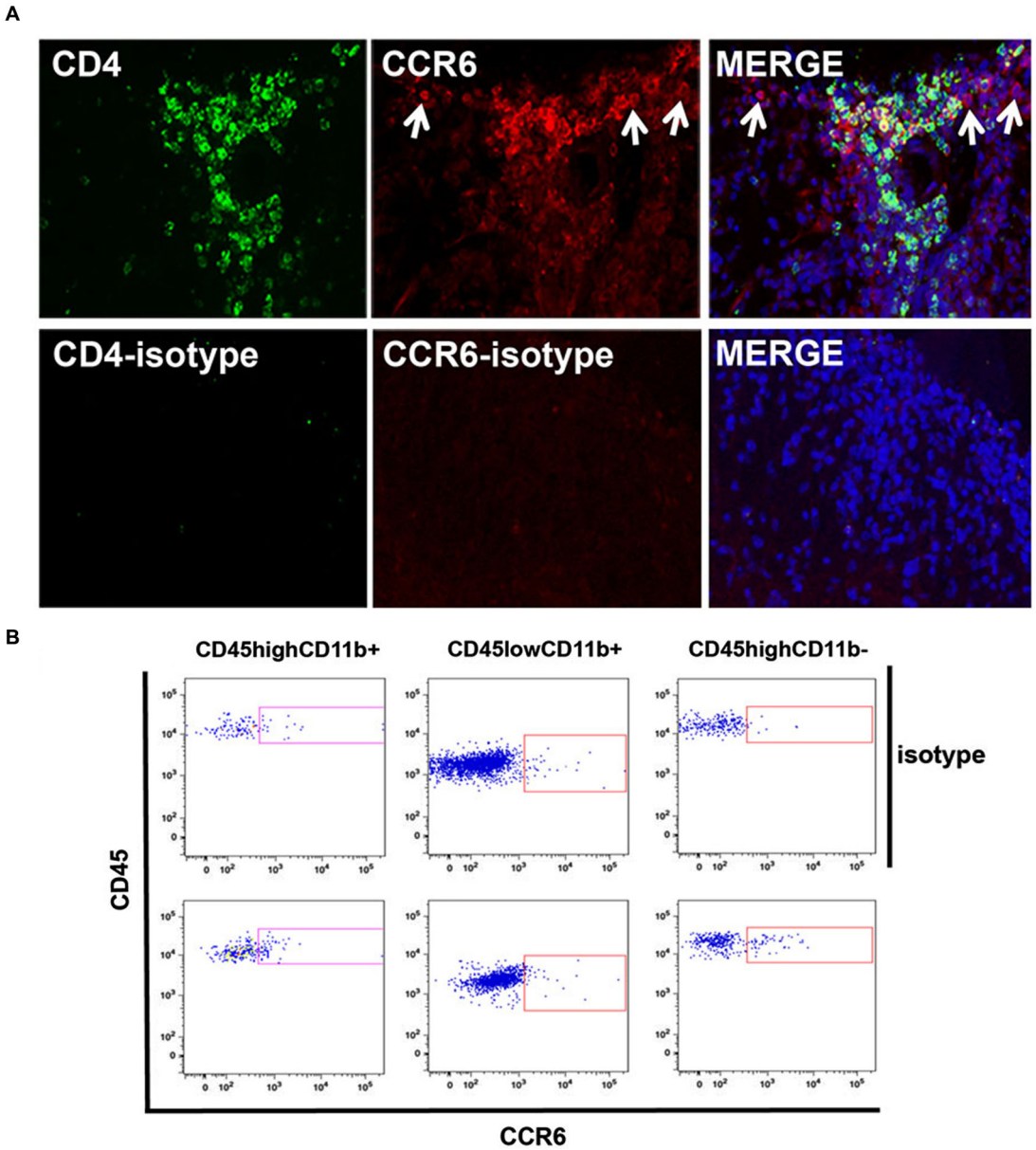

FIGURE 4 | Cellular localization of CCR6 expression in CNS. (A) CCR6 expression colocalized with $CD4^+$ T cells in spinal cord infiltrates in EAE. CCR6 expression was detected on $CD4^+$ cells by immunofluorescence microscopy in subpial infiltrates in EAE spinal cord. $CD4^+$ cells that lacked CCR6 expression can also be seen. CCR6 expression was also detected in cells that lacked CD4 expression (arrows). Micrographs are representative of tissue from 4 mice. Non-specific staining was evaluated by replacing CD4 and CCR6 antibodies with isotype-matched negative control antibodies. **(B)** Flow cytometry analysis of CCR6 expression by $CD45^{high}CD11b^+$, $CD45^{low}CD11b^+$ and $CD45^{high}CD11b^-$ cells in spinal cord of MOGp35-55-immunized mice with EAE. Profiles show CD45 and CCR6 (bottom panels) or isotype control (top panels) staining on cells gated by relative CD11b expression.

2010). IL-17 and downstream Act1 signaling enhanced TNF α-induced CCL20 expression in astrocytes (Kang et al., 2010), which could facilitate the entry of CCR6 expressing T cells into the CNS.

The leukocytic infiltrate in EAE is heterogeneous and includes, as well as T cells that are not specific for the disease-inducing immunogen MOG, macrophages, neutrophils and DC. CCR6 is expressed by many cell types, including B cells, T cells, DC, neutrophils and macrophages (Wojkowska et al., 2014 and reviewed in Lee et al., 2013). Th17 and regulatory T cells have come under the spotlight as $CCR6^+$ cells that play an important role in MS and EAE (Reboldi et al., 2009). Identification of $CD4^+$ T cells which do not express CCR6 in an inflammatory context is therefore of interest. A defining characteristic of chemokine immunology is redundancy, so suggestion that a particular receptor or chemokine ligand would not be essential

might not seem all that informative. However, the CCR6-CCL20 receptor-ligand pair is unusual in being non-redundant so neither can be substituted by other receptors or ligands, in the context of the paired interaction. Whether other receptor-ligand pairs can substitute for functional outcome then becomes a question. A recent study showed that CNS-infiltrating Th17 expressed CXCR2 (Wojkowska et al., 2014). Whether there is an absolute requirement for CCR6 for Th17 entry to the CNS has not been resolved.

One potential issue for interpretation of chemokine receptor analyses is that receptor ligation by chemokine may have led to downregulation of the receptor. We cannot exclude that this may have occurred and that the actual proportion of CNS-infiltrating $CCR6^+$ Th17 may have been higher than we estimated. However, since relatively low but comparable (>25%, <50%) proportions of any cytokine subset expressed CCR6, this argues against all of these T cells depending on CCR6 for their entry to the CNS, as well as against subset-specific dependence. Furthermore we show no $CCR6^+$ $CD8^+$ T cells, although all of them had infiltrated, and in this and another study we have shown that all of the $CD8^+$ and significant proportions of $CD4^+$ (of any cytokine subset) express CXCR3. We did not pursue the role of $CXCR3^+$ T cells further, and studies of the role of $CXCR3^+$ T cells in EAE continue to yield quite divergent findings (Liu et al., 2005; Muller et al., 2010; Sporici and Issekutz, 2010; Lalor and Segal, 2013). Our data does not exclude that CCR6-negative T cells had once expressed CCR6. Despite the potential for downregulation of CCR6 expression by T cells following encounter with CCL20, we show that Th1 as well as mixed Th1+Th17 do express CCR6.

There is a divergent literature on the role of CCR6 in EAE. Adoptive transfers showed that CCL20 was not required for the effector phase of EAE, although neutralizing antibodies reduced disease severity (Kohler et al., 2003). Mice deficient in CCR6 or treated with blocking antibodies, although relatively resistant to EAE, nevertheless developed mild disease (Liston et al., 2009; Reboldi et al., 2009; Moriguchi et al., 2013). Other studies showed that mice lacking CCR6 actually developed more severe or chronic EAE, attributed either to reduced regulatory T cell recruitment (Villares et al., 2009), or lack of $CCR6^+$ $PDL1^+$ mDC (Elhofy et al., 2009). In all of the knockout studies, CCR6-deficient T cells infiltrated the CNS.

The predominance of IFNγ-secreting T cells in the CNS of mice with severe EAE is very striking. There have been conflicting reports on the role and requirement for IFNγ in EAE. This is the only cytokine to have been directly shown to be pro-pathogenic in MS (Panitch et al., 1987), although that is not necessarily a desirable or easily achievable demonstration for other cytokines. Recent papers have provided a more nuanced perspective on the role for IFNγ in EAE and MS, showing that timing and possibly location of expression influence outcome of its expression (Hindinger et al., 2012; Naves et al., 2013). The mixed Th1+17 subset is a prominent and consistent feature of our analyses of MOG-induced EAE and has been implicated in MS (Kebir et al., 2009). It has been reported that polarized Th17 can convert to IFNγ-producing T cells *in vivo* (Shi et al., 2008; Bending et al., 2009; Lee et al., 2009). One of the roles recently identified for IFNγ is controling recruitment of Th17 (Berghmans et al., 2011), which increases interest in the Th1+17 subset. The previously bipolar debate on the relative roles of Th1 versus Th17 in EAE is given broader perspective by such considerations. Also, it is now clear that neither of the nominal cytokines for Th1 or Th17 are themselves necessary for EAE, but a third cytokine GM-CSF plays a key role (Kroenke et al., 2010; Codarri et al., 2011). We show that this cytokine is produced by $CD4^+$ and $CD8^+$ T cells and that as for IFNγ and IL-17, there is no obvious correlation with expression of the CCR6 or CXCR3 chemokine receptors. Our study has not attempted to differentiate between whether these cytokines are necessary or sufficient for disease, but does not support that the CCR6 chemokine receptor aligns with any of them. Expression by macrophages points to the possibility of their interaction with CCL20-producing astrocytes, an aspect that deserves further attention. It cannot be excluded that some microglia may also express CCR6.

The importance of CCR6 signaling for induction of EAE is shown by disease reduction in mice lacking this receptor, and by studies in which the receptor or its CCL20 ligand were blocked (Kohler et al., 2003; Liston et al., 2009; Reboldi et al., 2009). A question that arises is whether this receptor selectively controls entry of Th17 to the CNS. Findings from direct analysis of T cells that had entered the CNS in established EAE do not support this, nor do they support any association with GM-CSF producing cells. Similarly this and another study do not support association of CXCR3 with Th1 or IFNγ-producing T cells (Mony et al., 2014). Importantly our analyses also identify T cells that may not express either of these chemokine receptors. This points to there being a wider spectrum of chemokine responses driving EAE rather than only CCR6 and CXCR3. One candidate pathway involves CCR2, which has three potential ligands, although it is more implicated in regulation of macrophage entry. Other receptors such as CCR8 have also been implicated, especially in TNF-driven induction of glial response at the blood-brain barrier (Mürphy et al., 2002), as well as CXCR2 (Wojkowska et al., 2014). It has been reported that Th17 can co-express CCR4 and CCR6 (Mehling et al., 2010), but EAE (with reduced severity) could be induced in a double knockout mouse, and CCR6-negative $CD4^+$ T cells infiltrated the CNS (Moriguchi et al., 2013). The possibility of substitution by other receptor-ligand interactions might help explain lack of black-vs-white findings from ablation or blockade of selected chemokines or their receptors.

We have used direct analyses to show lack of alignment between chemokine receptors with T cell cytokine subsets in the inflamed CNS. This highlights challenges for development of chemokine-directed therapy for MS, and underlines the elegance, complexity and tremendous importance of chemokines in controling immunesurveillance as well as pathophysiologic T cell entry to the CNS.

AUTHORS AND CONTRIBUTORS

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Chemokines in the balance: maintenance of homeostasis and protection at CNS barriers

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In the adult central nervous system (CNS), chemokines and their receptors are involved in developmental, physiological and pathological processes. Although most lines of investigation focus on their ability to induce the migration of cells, recent studies indicate that chemokines also promote cellular interactions and activate signaling pathways that maintain CNS homeostatic functions. Many homeostatic chemokines are expressed on the vasculature of the blood brain barrier (BBB) including CXCL12, CCL19, CCL20, and CCL21. While endothelial cell expression of these chemokines is known to regulate the entry of leukocytes into the CNS during immunosurveillance, new data indicate that CXCL12 is also involved in diverse cellular activities including adult neurogenesis and neuronal survival, having an opposing role to the homeostatic chemokine, CXCL14, which appears to regulate synaptic inputs to neural precursors. Neuronal expression of CX_3CL1, yet another homeostatic chemokine that promotes neuronal survival and communication with microglia, is partly regulated by CXCL12. Regulation of CXCL12 is unique in that it may regulate its own expression levels via binding to its scavenger receptor CXCR7/ACKR3. In this review, we explore the diverse roles of these and other homeostatic chemokines expressed within the CNS, including the possible implications of their dysfunction as a cause of neurologic disease.

Keywords: chemokines, central nervous system, blood brain barrier, homeostasis, vasculature, choroid plexus, meninges, neurogenesis

INTRODUCTION

Our understanding of the role of chemokine expression in the adult central nervous system (CNS) has shifted away from viewing these molecules primarily as proinflammatory mediators and more towards their ability to exert neuroprotective and reparative functions. This is especially the case for chemokines categorized as "homeostatic", based on their constitutive expression in thymic and lymphoid tissues (CCL14, CCL19, CCL20, CCL21, CCL25, CCL27, CXCL12 and CXCL13), where they regulate the migration of leukocytes during immune surveillance (Bachmann et al., 2006; Ito et al., 2011). Of these chemokines, CCL19, CCL20, CCL21, CCL27 and CXCL12 are expressed within uninflamed CNS tissues (van der Meer et al., 2000; Stumm et al., 2002), particularly at CNS endothelial barriers (Kivisäkk et al., 2004; Reboldi et al., 2009). Two additional chemokines, CX_3CL1 and CXCL14, are also expressed at high levels in the normal CNS, primarily by neurons (Harrison et al., 1998; Huising et al., 2004; Banisadr et al., 2011). Ligands as well as receptors for several CNS homeostatic chemokines are expressed by neural stem cells (Huising et al., 2004; Kokovay et al., 2010), while others can be found on microglia and neurons (Sheridan and Murphy, 2013). These chemokines and their receptors are therefore involved in a range of homeostatic processes including immune surveillance, neuro/gliogenesis and modulation of synaptic transmission. This review will discuss how homeostatic chemokines protect and maintain normal CNS functions.

CHEMOKINES REGULATE CNS IMMUNE PRIVILEGE AND SURVEILLANCE

CNS BARRIERS

Homeostasis of the CNS is maintained within strict limits by anatomical and immunological barriers that restrict access of pathogens, solutes, and to an extent, immune cells, to the brain parenchyma. This review focuses on two of these barriers in particular; the blood–cerebrospinal fluid (CSF) barrier and blood brain barrier (BBB), which prevent the exchange of cells and solutes between the blood and CSF or brain parenchyma, respectively. The BBB is comprised of specialized endothelial cells of the cerebral microvasculature, surrounding pericytes, and astrocytic endfeet, while the blood–CSF barrier is largely made up of the fenestrated endothelium of the choroid plexus. In addition to these anatomical barriers, the expression of chemokines and chemokine receptors at the BBB and blood-CSF barrier serves as an immunological checkpoint and prevents (during non-inflammatory/homeostatic conditions) or promotes (during

neuroinflammation) the infiltration of circulating leukocytes into the deeper CNS parenchyma and ventricular or subarachnoid CSF spaces (**Figure 1**).

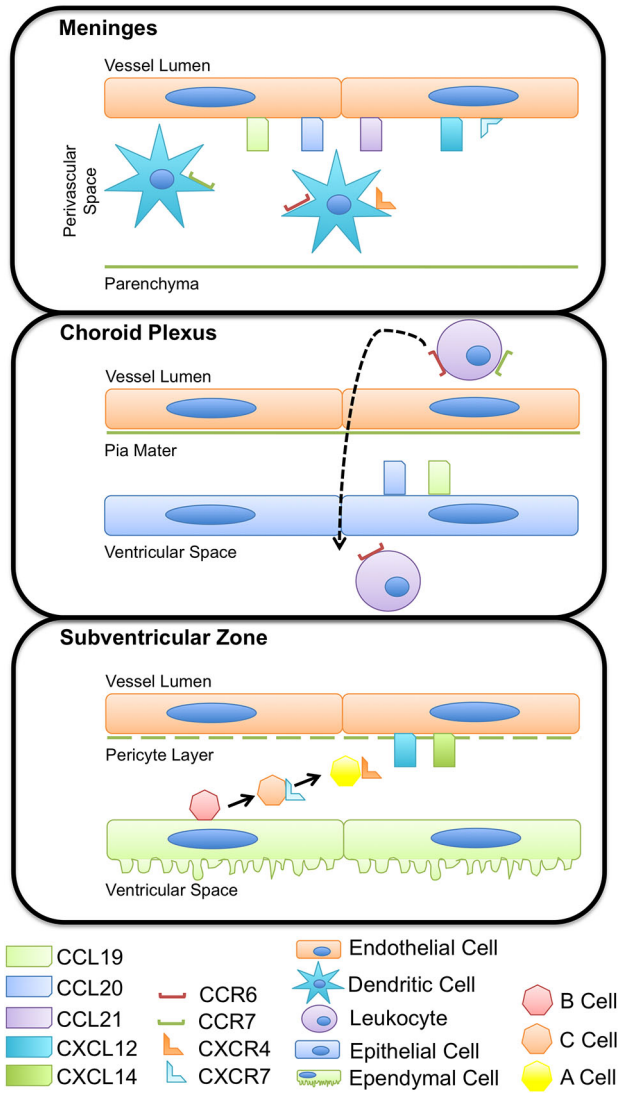

FIGURE 1 | Chemokines regulate immune and progenitor cell homeostasis at multiple CNS barriers. Chemokines expressed by the CNS vasculature have a role in regulating immune cell and neural progenitor cell occupancy within perivascular spaces. Expression of CCL19, CCL20, and CCL21 on meningeal vessels maintain dendritic cell populations important for immunosurveillance within the boarders of the glia limitans, limiting antigen presentation cell access to the CNS parenchyma. The vasculature of the choroid plexus expresses a variety of adhesion molecules and chemokines, including CCL19 and CCL20 that influence immune cell adhesion, rolling, and extravasation across the endothelium and pia mater. $CCR6^{+}$ and $CCR7^{+}$ leukocytes enter CSF-containing ventricles and circulate through the CNS, surveying for antigen and other cues indicative of inflammation. Subventricular zone (SVZ) neural precursors receive external cues, namely CXCL12, from the vasculature, that prompts proliferation, differentiation, and migration. Ependymal cells form a thin epithelial-like lining of the ventricular system and enclose the niche in which SVZ progenitors thrive.

As in peripheral lymphoid tissues, expression of chemokines and their cognate receptors within the CNS are highly regionalized and regulated in a tissue-dependent manner (Réaux-Le Goazigo et al., 2013). In particular, endothelial cell chemokines expressed at the BBB can translocate from the abluminal to luminal surfaces of post-capillary venules, thereby exerting effects on circulating leukocytes. Thus, a critical aspect of chemokine function at the BBB, as in peripheral tissues, is their localization along endothelial cell surfaces and binding to extracellular matrix proteins and glycosaminoglycans (GAGs). The discovery of chemokines' ability to direct leukocyte migration was largely informed through *in vitro* studies of the movement of leukocytes towards increasing concentrations of solubilized chemokines (Zachariae, 1993). These analyses perhaps inappropriately fostered the notion that within tissues, leukocytes similarly respond to soluble chemokine gradients; however it is now recognized that the extracellular matrix and GAGs localize and concentrate chemokines, preventing their rapid diffusion and loss of chemotactic effects (Hamel et al., 2009). In particular, chemokines have been shown to bind with high affinity to heparan sulfate chains of heparan sulfate proteoglycans, immobilizing chemokines and leading to the formation of chemokine gradients on endothelial surfaces (Johnson et al., 2005; Parish, 2006).

The concept of immune privilege was originally conceived as a result of experiments that found antigenic material, including foreign tumors and tissue grafts, failed to elicit a systemic, T cell-mediated immune response when implanted into the CNS parenchyma (reviewed in Galea et al., 2007). While the term immune privilege implies an absence of immunological response within the CNS, it is now recognized that CNS immune privilege is not absolute but rather very elaborately controlled. Several cellular and molecular components that comprise the CNS barriers are responsible for limiting the immune response under homeostatic conditions.

Microglia and perivascular macrophages are critical components of CNS immune surveillance and protection. While microglia share morphology and many functions with perivascular macrophages, their ontogeny differs. Microglia are myeloid phagocytes (Nimmerjahn et al., 2005) derived from the yolk sac during early development (Ginhoux et al., 2010; Kierdorf et al., 2013) and are found throughout the CNS of adults. Once activated, microglia initiate a classical innate immune response, similar to that elicited by peripheral macrophages, and facilitate activation of adaptive immunity, secreting inflammatory cytokines and presenting antigen(s) to reactive lymphocytes. In adults, perivascular macrophages originate from stem cell niches in the bone marrow and localize to perivascular spaces, confined by the BBB around blood vessels, via the circulation (Neumann and Wekerle, 2013). Perivascular macrophages function similar to peripheral macrophages, and thus they are crucial for antigen presentation to and reactivation of lymphocytes, making them a critical component of CNS-defense against invading pathogens. Further, elimination of these perivascular cells enhances responses to inflammatory stimuli, including LPS, suggesting that perivascular macrophages may have a role in controlling initial host-pathogen responses within the CNS (Serrats et al., 2010). While

a repertoire of chemokine and chemokine receptors are expressed by microglia and perivascular macrophages, their function with regard to antigen presentation is not well understood. However, it is likely that chemokines underlie the localization of these cells within their respective CNS compartments. Together, microglia and perivascular macrophages form another layer of CNS protection, comprising a cellular barrier to facilitate protection against invading pathogens as well as immune-mediated bystander CNS injury.

THE CHOROID PLEXUS AND MENINGEAL BARRIERS

The choroid plexus and the meninges represent pivotal modified cellular barriers between the blood and CSF or parenchymal compartments, respectively. The choroid plexus is largely considered a circumventricular organ, localized in the ventricles, and constitutes one of the interfaces between the blood and the CSF (Strazielle and Ghersi-Egea, 2000; Schulz and Engelhardt, 2005). The epithelial cells of the choroid plexus secrete CSF and thus largely contribute to brain homeostasis, adjusting intracranial volume, buffering extracellular solutes, and supplying cells of the CNS with micronutrients (Strazielle and Ghersi-Egea, 2000). The vascular endothelial cells of the choroid plexus are unique from those of the BBB as they lack tight junctions, more readily enabling diapedesis of cells. Further, as opposed to postcapillary venules within the parenchyma, which require cells to traffic across two basement membranes, meningeal capillaries have only a one-layer structure (Wilson et al., 2010). In addition to representing points of access for circulating immune cells, the choroid plexus and the meninges also host a local population of antigen presenting cells. In rats and humans, choroid plexus- and meninges-associated dendritic cells (DCs) have been identified, which express major histocompatibility class II (MHC II) and present antigen to circulating lymphocytes (McMenamin, 1999). These DCs are known to sample the environment by extending processes between adjacent choroid plexus epithelial cells (Serot et al., 2000), making the choroid plexus and the meninges major sites of immunosurveillance (**Figure 1**).

The localization of DCs in close proximity to the vessels of the choroid plexus and meninges suggests that these cells express a set of chemokines that limit their mobility out of these compartments. Further, DC turnover dictates that these cells exhibit temporal expression of chemokine localizing cues to facilitate their egress from the circulation and into the choroid plexus and meningeal compartments (Chinnery et al., 2010). DCs are known to express several chemokine receptors including CXCR3, CCR6, CCR7, and CXCR4 as well as some others, depending on their stage of maturation (Charles et al., 2010), and numerous chemokines have been identified in recruiting DCs into the brain parenchyma during neuroinflammation as well as prion disease, viral encephalitis, brain ischemia, parasitic and bacterial CNS infections (Clarkson et al., 2012). However, it remains unclear which chemokines are involved in maintaining DCs within CNS compartments during immune surveillance. Under homeostatic conditions, the presence of DCs in the stroma of the chroroid plexus as well as meningeal blood vessels suggest a role for chemokines that are constitutively expressed at these locations, such as CCL19, CCL20 and CCL21 (**Figure 2**). Recent studies have also demonstrated vessel-associated, DC-like cells in the CNS perivascular space that extend cell processes into the basement membrane of the glia limitans in the absence of inflammation. These DC-like cells expressed CD11c, but not MHC II (Prodinger et al., 2011). The authors of this study speculate that these cells may represent a subpopulation of microglia, cells of the monocytic lineage, or immature or quiescent DCs capable of recognizing and presenting antigen. Since these cells extend processes into the glia limitans, it is possible that they are capable of sampling and presenting antigen within the perivascular space. It is possible that abluminally expressed CXCL12, which binds to CXCR4 expressed by DCs, at the BBB may be important in retaining these cells within close proximity to the microvasculature, allowing interactions with surveying T cells, but preventing access to deeper parenchymal tissue.

Given that mature DCs up-regulate expression of cognate chemokine receptors, including both CCR7 and CXCR4 (Sallusto et al., 1998), it is possible that expression of these receptors localize mature DCs to the chroroid plexus (via CCL21) and BBB (via CCL19 and CXCL12), thus facilitating interactions between circulating lymphocytes and these antigen presenting cells. Antigen presented by mature DCs and recognized by T cell receptors (TCRs) results in heterodimerization between TCRs and CXCR4 and is necessary to initiate activation, cytokine secretion, and T cell migration. Further, CXCL12 has been shown to enhance T cell responses via costimulation of the TCR (Smith et al., 2013), suggesting that CXCL12 expressed in the perivascular niche may play an important role in mediating TCR activation during antigen presentation. Furthermore, heterodimerization of CXCR4 and TCR (Kremer et al., 2011) triggers TCR signaling via β-arrestin-1 that results in down-regulation of CXCR4 (Schneider et al., 2009; Fernández-Arenas et al., 2014), perhaps limiting CXCL12 action at the BBB and preventing prolonged retention of T cells within the perivascular space (**Figures 1** and **2**).

The chemokine CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is expressed as three alternatively spliced isoforms (α, β, and γ). Within the CNS, the expression patterns for CXCL12 is widespread and includes the cortex, olfactory bulb, hippocampus, cerebellum, meninges, and the endothelium of the BBB (β and γ isoforms). Further, expression of CXCR4, the receptor for CXCL12, has been detected in numerous cell types in the CNS including astrocytes, microglia, oligodendrocytes, neurons, and endothelial cells of the BBB (van der Meer et al., 2000; Stumm et al., 2002). Until recently it was believed that CXCL12 mediated its effects exclusively via interactions with CXCR4, however the receptor CXCR7/ACKR3 (Atypical chemokine receptor 3), formerly the orphan receptor RDC1, has now been shown to bind CXCL12 as well as CXCL11 (Burns et al., 2006). While CXCR7/ACKR3 (CXCR7) possesses homology with conserved domains of G-protein coupled receptors and is structurally similar to other CXC receptors, ligand binding does not initiate typical intracellular signaling pathways but instead results in β-arrestin recruitment and MAP kinase activation (Rajagopal et al., 2010; Odemis et al., 2012). One function of CXCR7 appears to be its ability to act as a scavenger receptor for both CXCL12 and CXCL11, mediating uptake and degradation

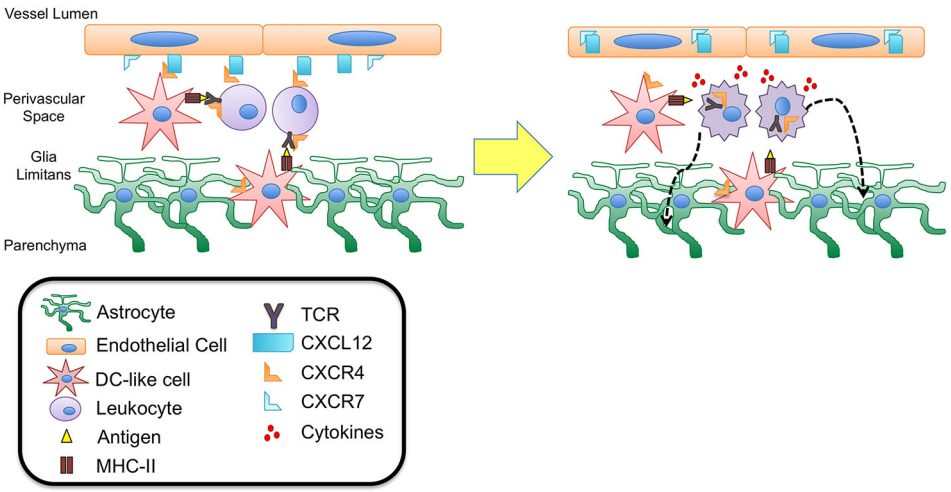

FIGURE 2 | CXCL12 fate determination of perivascular T cells. DC-like cells have been identified in the perivascular space as well as in the juxtavascular parenchyma abutting astrocytic end feet and extending processes into the basement membrane of the glia limitans. These cells express CD11c but lack MHC II expression suggesting they may be quiescent DCs capable of recognizing antigen during routine immune surveillance. It is possible that upon antigen uptake these cells are activated to up-regulate MHC II expression as well chemokine receptors such as CXCR4. Interaction with CXCL12 expressed at the abluminal side of the BBB endothelium could thus function to retain these cells as well as circulating lymphocytes within the perivascular spaces, facilitating antigen presentation and recognition. Upon binding to antigen presented in MHC II complexes, the T cell antigen receptor (TCR) expressed on T cells heterodimerizes with CXCR4, initiating intracellular signaling cascades that lead to T cell activation and migration. Recent studies have demonstrated that TCR-activated β-arrestin signaling down-regulates T cell surface CXCR4 expression, perhaps removing an important retention cue within the perivascular niche, thereby promoting entry of T cells into deeper parenchymal tissue. This process may also be promoted by the release of cytokines due to TCR binding and T cell activation. Inflammatory cytokines have been shown to up-regulate levels of CXCL12 as well as the scavenger chemokine receptor CXCR7. CXCR7 is capable of binding to and internalizing CXCL12, leading to loss of polarity at the BBB. Relocation of CXCL12 from abluminal to luminal endothelial surfaces at the BBB has been shown to lead to extensive leukocyte infiltration and neuroinflammation in experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS).

of these ligands, and thus regulating extracellular chemokine concentrations (Boldajipour et al., 2008; Naumann et al., 2010). The ability of CXCR7 to act as a sink for CXCL12 may have implications for controlling chemokine gradients and directing hematopoetic cells, leukocytes and other cell subsets to peripheral lymphoid tissues as well as the CNS. CXCR7 expression within the CNS of rats was detected via *in situ* hybridization, with CXCR7 mRNA transcripts identified in the ventricular ependyma, the choroid plexus, neuronal and astroglial cells as well as cells of the vasculature (Schönemeier et al., 2008a,b). Interestingly, studies have identified the endothelium of the BBB as a source of constitutive expression of CXCL12 and CXCR4 as well as CXCR7, suggesting a role for this chemokine/receptor axis in regulating immune cell trafficking at the BBB during homeostasis.

These initial observations of CXCL12/CXCR4 expression by BBB endothelial cells were expanded by McCandless et al. who demonstrated the importance of CXCL12 expression and polarization at the BBB in the perivascular localization of infiltrating mononuclear cells (McCandless et al., 2006). These studies determined that CXCL12 protein is normally localized along the abluminal surface of endothelium within the CNS of mice and humans. During the autoimmune diseases experimental autoimmune encephalomyelitis (EAE) in mice and multiple sclerosis (MS) in humans, CXCL12 localization shifts toward a more luminal expression pattern, which is accompanied by increased parenchymal entry of CXCR4 positive mononuclear cells. Loss of CXCL12 polarity and luminal display of this chemokine was also associated with the detection of activated CXCR4 on leukocytes within the blood, suggesting that relocation of CXCL12 in this manner not only promotes the egress of leukocytes from perivascular spaces but also increases their capture and translocation across the BBB. These results suggest that abluminal expression of CXCL12 at the CNS vasculature during homeostatic conditions is a component of immune privilege essential for limiting extravasation of circulating leukocytes across endothelial barriers, while restricting immune cells to the perivascular space, limiting their access to parenchymal tissues (**Figure 2**).

CHEMOKINE SIGNALING AT CNS BARRIERS IN HEALTH AND DISEASE

The CXCL12, CXCR4, CXCR7 axis

More recently, the role of CXCR7 in regulating CXCL12 polarity at the BBB was examined by Cruz-Orengo et al. (2011). Consistent with earlier reports (Schönemeier et al., 2008a,b), results from this study found constitutive CXCR7 expression by the CNS vasculature. In addition, CXCR7 message was detected in primary cultures of murine brain microvascular endothelial cells (BMECs). During EAE, CXCR7 levels increased at postcapillary venules of spinal cord white matter, with concomitant loss of CXCL12 polarity at the BBB (**Figure 2**). Administration of an antagonist to the CXCR7 receptor prevented loss of

abluminal CXCL12, limiting leukocyte entry at the BBB and the formation of parenchymal inflammatory lesions. *In vitro* experiments using murine BMECs determined that proinflammatory T cell cytokines, interleukin (IL)-1β and IL-17, increased expression of CXCL12 and CXCR7, respectively, with increased localization of CXCL12 with lysosomal markers. Further, these cytokines enhanced uptake of exogenous CXCL12, which was inhibited in a dose-dependent manner by antagonism of CXCR7, suggesting that under inflammatory conditions, CXCR7 facilitates internalization of CXCL12, leading to loss of polarity at the BBB seen in EAE as well as MS (Cruz-Orengo et al., 2011).

Taken together, these results suggest a critical role for CXCR7 in mediating CXCL12 abundance and localization during neuroinflammation, but importantly may offer clues to the role of this chemokine/receptor axis during homeostasis. In the absence of inflammation, CXCL12 expression is localized on the abluminal surface of the BBB, despite constitutive expression of CXCR7 at the CNS microvasculature. This perhaps indicates that CXCR7 normally functions to maintain and replenish basal CXCL12 through internalization and recycling to the cell surface, as has been demonstrated in cell lines engineered to express CXCR7 (Luker et al., 2010) and in studies of germ cell migration (Mahabaleshwar et al., 2012). At this time, it is unclear if CXCR7 plays a similar role in mediating extravasation of immune cells across the BBB, however the maintenance of CXCL12 polarity during homeostatic conditions indicates that this mechanism may be an essential function of CXCR7 in non-pathogenic states.

CCL2 and CCR2

CCL2 is also known as monocyte chemoattractant protein-1 (MCP-1), an inflammatory chemokine expressed by immune cells as well as other stromal cell types. In response to inflammatory cues or tissue injury, CCL2 is up-regulated to recruit $CCR2^+$ monocytes, memory T cells, and DCs (Kolattukudy and Niu, 2012). Within the CNS, CCL2 is expressed by neurons, astrocytes, and microvascular endothelial cells of the BBB, and the role of CCL2 in recruiting monocytes and macrophages into the CNS under inflammatory conditions has been well characterized (Conductier et al., 2010; Réaux-Le Goazigo et al., 2013). Nevertheless, recent studies have also demonstrated a role for CCL2 under homeostatic conditions and suggest that the expression of both CCL2 and CCR2 is necessary for perivascular and meningeal macrophage recruitment and turnover in the brains of mice (Schilling et al., 2009). Additionally, Stowe et al. have shown that hypoxic preconditioning of mice with 8% oxygen for 4 h led to an up-regulation of CCL2 by neurons as well as cerebral endothelial cells that was associated with increased tolerance to subsequent cerebral ischemia. This study also found that hypoxic preconditioning and up-regulation of CCL2 at the cerebral microvasculature was not sufficient to increase monocyte trafficking across the BBB (Stowe et al., 2012). These results suggest that CCL2 levels on microvascular endothelial cells can be up-regulated in the absence of inflammation and may function to confer a neuroprotective phenotype at the BBB.

LEUKOCYTE HOMING IN CEREBROSPINAL FLUID

The intensity of immune responses in the CNS increases with proximity to the ventricles of the brain (Matyszak and Perry, 1996), and materials implanted within the subarachnoid space and meninges are likewise capable of eliciting a robust immune response. These observations suggest that the ventricular and subarachnoid CSF may function as sites of physiological immune surveillance. Consistent with this, cellular infiltrates that accumulate within the meningeal membranes during neuroinflammatory events have been observed to arrange in formations resembling secondary lymphoid structures (Howell et al., 2011), while infiltrates within the parenchyma do not exhibit the features of lymphoid neogenesis.

The CCL19, CCL21, and CCR7 axis

In peripheral lymphoid tissues, the chemokine CCL19 guides CCR7-expressing B cells, naïve T cells, and DCs into lymphoid tissue under physiological conditions (Bachmann et al., 2006). Along with the chemokine, CCL21, CCL19 is constitutively expressed in lymphoid tissues including the spleen, Peyer's patches, and lymph nodes, where they regulate homing of leukocytes (e.g., $CCR7^+$ naïve T cells and mature DCs) and facilitate antigen-specific interactions within subcompartments of secondary lymphoid tissue (e.g., T cell zones and high endothelial venules). Thus CCL19 and CCL21 serve to generate adaptive immune responses and are critical for developing and maintaining secondary lymphoid tissues in the periphery and have also been implicated in lymphoid neogenesis within the CNS.

Kivisäkk et al. have shown that $CD4^+$ T cells are restimulated within the subarachnoid space by encounters with MHC II^+ antigen presenting cells prior to the onset of inflammation in EAE, providing further support to the concept of the subarachnoid space and meninges as a site of routine immunological surveillance (Kivisäkk et al., 2003, 2009). Studies from this group have also characterized the phenotype of leukocytes in the CSF of patients without CNS inflammation and found that these cells predominantly are $CD4^+/CD45RA^-/CD27^+/CD69^+$, consistent with the profile of activated central memory T cells that also express high levels of CCR7 and L-selectin. Given that CCL21, a ligand for CCR7, has been detected at the choroid plexus epithelium (Kivisäkk et al., 2004), it is possible that this chemokine directs $CCR7^+$ activated memory T cells to cross the blood-CSF barrier during homeostatic immune surveillance of the CNS (**Figure 1**). The expression of CCR7 by these memory T cells also suggests that this chemokine receptor is important in maintaining these lymphocytes within the CNS, perhaps via in interactions with CCL19 or CCL21 expressed at the brain vasculature during physiologic as well as neuroinflammatory conditions. Additionally, the expression of CCR7 by memory T cells within the CSF may facilitate homing back to peripheral lymphoid tissue, particularly the deep cervical lymph nodes via drainage of the CSF across the cribriform plate and nasal mucosa (Goldmann et al., 2006; Laman and Weller, 2013).

CCL19 mRNA transcripts are constitutively expressed on the endothelial cells of post-capillary venules in the brain and spinal cord under physiologic conditions, while expression of CCL21, another CCR7 ligand, is induced at post-capillary venules

only during neuroinflammation (Alt et al., 2002; Krumbholz et al., 2007). CCL19 transcripts were also detected in normal human brain homogenates while expression levels were elevated in homogenates from active and inactive MS lesions. Kivisäkk et al. examined the expression of CCR7, CCL19, and CCL21 in brain autopsy material and CSF samples from MS patients. In contrast to previous observations in mice, this study reported a lack of CCL19 or CCL21 protein expression in endothelial or parenchymal cells of non-lesioned white matter or in active or chronic MS lesions, but did find strong CCL21 immunoreactivity within the choroid plexus epithelium (Kivisäkk et al., 2004). The expression of these lymphoid chemokines in the CNS implies that CCL19, and to a lesser extent CCL21, may signal circulating leukocytes that normally home to peripheral lymphoid tissues. Based on the constitutive expression of CCL19 within the CNS, it is possible that this lymphoid chemokine may also function in physiological immune surveillance of the CNS, perhaps at the level of the postcapillary venules of the BBB by recruiting and retaining T cells, as well as other cells known to express CCR7 (e.g., B cells; **Figure 1**).

CCL20 and CCR6

The receptor CCR6 is unique among chemokine receptors in that it binds a single chemokine ligand, CCL20. In the periphery, CCR6 regulates mucosal immunity via several mechanisms, including mediating the recruitment of DCs to epithelial barriers during inflammation and homing of helper T cells and DCs to the mucosal lymphoid tissue of the gut (Ito et al., 2011). Accordingly, CCL20 is constitutively expressed at epithelial barriers of the skin, lungs, gut and choroid plexus, typically at low levels under non-pathologic conditions. However, in response to proinflammatory cytokines, CCL20 levels can be substantially up-regulated. In addition to recruiting leukocytes to mucosal barriers, recent experiments have implicated CCL20/CCR6 in the trafficking of T cells to the CNS across the choroid plexus during immune surveillance as well as neuroinflammation (**Figure 1**). Reboldi et al. demonstrated that CCL20 was constitutively expressed by the choroid plexus epithelium in both mice and humans, but was not expressed by endothelial cells of the parenchymal microvessels. Mice lacking CCR6, which is expressed on IL-17-producing T cells (Th17 cells), were highly resistant to active induction of EAE by MOG immunization. Further, in these CCR6-deficient mice, $CD45^+$ cells accumulated within the parenchyma of the choroid plexus, but failed to enter the CNS. Passive transfer of EAE into CCR6 knockout mice using MOG-specific WT T cells was able to rescue disease susceptibility and led to recruitment of T cells, including those lacking CCR6, into the CNS parenchyma. This finding suggests that the initial trigger for inflammation in this EAE model was due to a CCR6-dependent entry of Th17 cells into the uninflamed CNS via trafficking across the CCL20-expressing choroid plexus epithelium, leading to a second wave of infiltration of lymphocytes that does not rely on CCR6 (Reboldi et al., 2009). The results from this study led these authors to speculate that CCL20/CCR6 is critical for surveillance of the CNS via the CSF and subarachnoid spaces.

CCL20 and CCR6 may also play a role in the entry of autoreactive T cells during EAE at the dorsal blood vessels of the fifth lumbar vertebrae (L5) of the spinal cord via an IL-6-mediated mechanism. Arima et al. have demonstrated that CCL20 is normally expressed by vasculature at this site. IL-6 mediated upregulation of CCL20 was induced by stimulating the soleus muscle, which led to the infiltration of autoreactive T cells across the BBB in an adoptive transfer model of EAE. These experiments provided evidence that sensory stimulation can trigger chemokine-mediated accumulation of T cells within the CNS microvasculature at specific locations within the lumbar spinal cord. These observations led the authors to postulate that expression of CCL20 at the dorsal L5 blood vessels, even in the absence of pathogenic T cells, could represent a "gateway" by which leukocytes expressing CCR6 could cross the BBB and gain entry into the CNS parenchyma (Arima et al., 2012).

CHEMOKINES AND MAINTENANCE OF THE ADULT CNS

PROGENITOR AND ENDOTHELIAL CELL FUNCTION

At one time, loss of neurons was thought to be irreversible in the adult brain; we now know that generation of replacement cells is an ongoing process in rodents and humans (Kuhn et al., 1996; Eriksson et al., 1998). Adult neurogenesis occurs in localized "neurogenic niches" from precursors that reside adjacent to the lateral ventricles, in the subventricular zone (SVZ) and in the subgranular zone (SGZ) of the hippocampus (Gage, 2000; Doetsch and Scharff, 2001; Alvarez-Buylla and Lim, 2004; Zhao et al., 2008; Sanai et al., 2011). Stem cells require extracellular signals produced by the CNS milieu to regulate their ability to self-renew, proliferate, and differentiate (Sanai et al., 2011). Cues within these local microenvironments perpetuate new neurons and facilitate their integration into the existing brain circuitry (van Praag et al., 2002; Zhao et al., 2008). Neural progenitor cells (NPCs) give rise to adult neurons, astrocytes, and oligodendrocytes, and can be classified into several lineages. $GFAP^+$ astrocyte-like type B cells line the lateral ventricle and extend processes into the SVZ plexus blood vessels (Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008). During lineage progression, type B cells give rise to transit amplifying type C cells (Pastrana et al., 2009). Type C cells rapidly divide and give rise to type A neuroblasts, which proliferate as they migrate in chains along blood vessels (Shen et al., 2008; Tavazoie et al., 2008). The endothelial cells that make up the BBB vasculature serve as neurogenic "highways", mediating progenitor cell trafficking and differentiation by providing external signage as guidance cues.

The CNS endothelium is essential for maintenance and homeostasis of the neural progenitor pool. In the adult, bone marrow-derived endothelial progenitor cells (EPCs) express CXCR4 and respond to CXCL12 as well as other cytokines to augment neovascularization for restoration of homeostasis following CNS injury (Zhang et al., 2002; Zheng et al., 2007; Yamaguchi et al., 2003). These epithelial cells directly or indirectly give rise to all the neurons, astrocytes, and oligodendrocytes in the adult brain (Deverman and Patterson, 2009). Not only does the endothelium give rise to CNS progenitors, the neurogenic niche is localized around the vasculature. Approximately 47% of dividing progenitor (type B cells) and 46% of transit amplifying (type C) cells are

located within 5 microns of the endothelium (Shen et al., 2004, 2008). These progenitors directly contact the vessels of the SVZ in areas devoid of astrocyte end-feet and pericyte coverage, suggesting the vasculature endothelium, in particular, is an essential matrix and source of external cues for NPCs (Shen et al., 2008; Teng et al., 2008).

THE CXCL12/CXCR4/CXCR7 AXIS AND NEURAL PROGENITOR CELLS

Chemokines expressed by the vasculature in the adult CNS are dynamically or constitutively regulated to provide migratory, proliferative, or differentiation cues to neurons and glia (Deverman and Patterson, 2009). Similar to hematopoietic progenitor cells in the bone marrow, in which CXCR4-CXCL12 signaling maintains the progenitor pool (Sugiyama et al., 2006), proliferative SVZ progenitor cells home to endothelial cells in the CNS in a CXCL12- and CXCR4-dependant manner under physiologic conditions. Kokovay et al. demonstrated that in early progenitor and transit amplifying cells, epidermal growth factor receptor and α6 integrin is up-regulated downstream of CXCL12 binding, enhancing the ability of activated NPCs to bind laminin on the CNS vasculature (**Figure 3**). Further, they showed that CXCL12 regulates the migration of neuroblasts from the SVZ (Kokovay et al., 2010), suggesting that endothelial CXCL12 can regulate progenitor cell occupancy of and departure from the vasculature niche of the adult SVZ. Similarly, in the SGZ, Schultheiss et al. have recently demonstrated that neuronal-committed progenitor cells express CXCR4 and that CXCR4 is phosphorylated in a CXCL12-dependent fashion (**Figure 3**). Further, deletion of CXCR4 in NPCs of adult mice resulted in reduced neurogenesis, specifically, a reduction in $Sox2^+$ early progenitors, $NeuroD^+$ neuronal-committed progenitors, and $doublecortin^+$ immature neurons was observed (Schultheiß et al., 2013). Together, these studies suggest that CXCL12-mediated CXCR4 activation is required for maintenance of NPCs in neurogenic zones of the adult CNS.

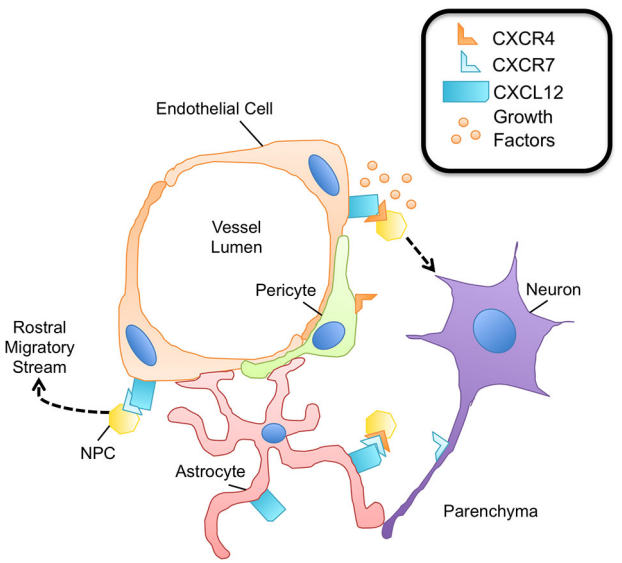

FIGURE 3 | The neurovascular unit promotes NPC survival. Within the neurovascular unit, multiple cell types promote the survival and maintenance of NPCs via expression of the CXCL12/CXCR4/CXCR7 chemokine axis. NPCs express both CXCR4 and CXCR7, capable of heterodimerization. Binding of this receptor complex to CXCL12 leads to NPC survival via phosphorylation of ERK1/2. The binding of CXCL12 to CXCR4 on NPCs promotes adhesion molecule expression for enhanced attachment of NPCs to endothelial cells, a rich source of growth factors important for NPC survival and proliferation. CXCR4 activation on NPCs is also critical to sustain the pool of neural-committed progenitor cells within neurogenic niches. Pericytes are thought to express CXCR4; however the downstream effects of CXCR4 signaling in pericytes are largely unknown. CXCR7 expression is known to contribute to the migratory capacity of NPCs, particularly in the context of entering the rostral migratory stream and traveling to the olfactory bulb. Axons have also been shown to express CXCR7 during development as well as during neuroinflammation, although its function is largely not understood.

Multiple pathways are known to regulate CXCR4 activation in neurogenesis. During development, chemokines position neural progenitors in the SGZ such that they are exposed to a range of neurogenic factors, including Wnt and Sonic hedgehog (Shh; Klein et al., 2001; Machold et al., 2003). These factors are critical for the maintenance of adult neurogenesis as Shh promotes proliferation of NPCs (Machold et al., 2003) and manipulation of the Wnt pathway nearly abolishes neurogenesis in the adult hippocampus (Lie et al., 2005). It is now known that CXCR4 is a downstream target of Wnt signaling, suggesting that Wnt induces CXCL12-mediated processes in NPCs via receptor regulation (Choe and Pleasure, 2012).

The alternate receptor for CXCL12, CXCR7, has also been shown to have a prominent role in adult neurogenesis. Zhu et al. demonstrated that both CXCR4 and CXCR7 are required for the survival of human NPCs. While CXCR4 is broadly expressed on the surface of human NPCs, CXCR7 was primarily localized in early endosomes, quickly trafficking to the plasma membrane to mediate CXCL12 endocytosis. Treatment of human NPCs with exogenous CXCL12, however, led to CXCR4/CXCR7 colocalization and downstream ERK1/2 signaling, which was shown to be essential for NPC survival (Zhu et al., 2012). In another study, CXCR7 expression regulated the migratory behavior of early neurons in the forebrain. CXCR7, but not CXCR4, was expressed by olfactory interneuron precursors, and down-regulation of CXCR7 impacted the ability of the precursors to integrate into the rostral migratory stream, the pathway to the olfactory bulbs (Tiveron et al., 2010). These studies suggest that CXCR7 has a prominent role in adult neurogenesis both in the context of and independent of CXCR4 signaling (**Figure 3**).

The CXCL12/CXCR4/CXCR7 axis has multiple roles in the homeostasis of adult neurogenesis. However, it is becoming increasingly clear that this axis may be up-regulated following injury including stroke (Hill et al., 2004; Wang et al., 2012), traumatic brain injury (Israelsson et al., 2008), or demyelination (Carbajal et al., 2010, 2011; Patel et al., 2010, 2012; Williams et al., 2014) to generate replacement cells and restore normal CNS function.

CXCL12 AND CXCL14 REGULATE HIPPOCAMPAL NEUROGENESIS

Following homology cloning, phylogenetic analysis revealed that CXCL14 is one of the oldest chemokines (Huising et al., 2004), yet

its functions are relatively unknown. Considered a homeostatic chemokine, CXCL14 (BRAK; breast and kidney derived) is constitutively expressed in many regions of the brain (Huising et al., 2004) including the cortex, basal ganglia, septum, hippocampus, and hypothalamus (Banisadr et al., 2011; Yamamoto et al., 2011). It is thought to have an opposing role to CXCL12 (Banisadr et al., 2011; Tanegashima et al., 2013) due to its ability to bind a shared receptor, CXCR4 (Tanegashima et al., 2013). CXCL14 is highly expressed in many regions of the adult brain, including the hippocampus, and may regulate synaptic inputs to adult NPCs (Banisadr et al., 2011). The early development of NPCs within the SGZ is regulated by excitatory GABAergic synaptic inputs that promote synaptic maturity (Ge et al., 2007; Ma et al., 2009). These newborn neurons mature and are synaptically integrated into the dentate gyrus of the hippocampus (van Praag et al., 2002). GABAergic synapses on adult NPCs are sensitive to CXCL12 and CXCL14, which enhance (Bhattacharyya et al., 2008) and inhibit the effects of GABA (Banisadr et al., 2011), respectively. These findings suggest that CXCL12 and CXCL14 work to regulate hippocampal integrity in mature mammals. This is consistent with experiments using a CXCR4 antagonist in adult mice where blockade of CXCR4 signaling impaired recognition and memory (Parachikova and Cotman, 2007). Taken together, these studies suggest that CXCL12 and CXCL14 have an opposing role, regulating NPC responses to synaptic stimulation, and maintaining balance in homeostatic NPC turnover in the adult brain.

CX_3CL1 MAINTAINS THE NEUROGENIC NICHE

CX_3CL1 (fractalkine) is much longer than most chemokines (373 vs. ∼80 AAs) and also exists in two forms: a 95 kDa membrane-bound form with an N-terminal chemokine domain, a glycosylated mucin-like stalk, a hydrophobic transmembrane region and an intracellular C-terminal domain; and a 70 kDa soluble form that contains only the N-terminal chemokine domain. The soluble chemokine domain of CX_3CL1, when cleaved, can act as a signaling molecule (Chapman et al., 2000), inducing chemotaxis in T cells and monocytes (Hermand et al., 2008), whereas its membrane-tethered mucin stalk can serve as a cell adhesion molecule, via binding of the CX_3CL1 receptor, CX_3CR1 (Haskell et al., 1999). Under physiologic conditions, CX_3CL1 is highly expressed by a variety of neurons throughout the CNS (Hatori et al., 2002), with especially high levels in hippocampal neurons (Sheridan and Murphy, 2013). Neurons and microglia both express its receptor, CX_3CR1 (Hatori et al., 2002), which regulates memory formation and synaptic plasticity via direct effects on glutamatergic synapses (Hoshiko et al., 2012; for an extensive review, see Sheridan and Murphy, 2013).

CX_3CL1 has been shown to play a key role in maintaining adult neurogenesis via indirect mechanisms that modify the CNS microenvironment. CX_3CL1 normally limits microglial activation and expression of proinflammatory cytokines including IL-1β, IL-6, and tumor necrosis factor (TNF)-α (Bachstetter et al., 2011; Rogers et al., 2011), which act directly on NPCs (Monje et al., 2003; Iosif et al., 2006; Koo and Duman, 2008). Thus, both genetic and antibody-based blockade of CX_3CR1 signaling attenuates the inhibition of microglial activation and impacts hippocampal neurogenesis in adult animals. With age, there is an increase in activated microglia, which can promote an inflammatory milieu (Gemma et al., 2007) and contribute to age-related declines in neurogenesis (Rao et al., 2006; Ben Abdallah et al., 2010). Both exogenous CX_3CL1 or IL-1R antagonist reverse this decline in aged animals. In addition, cleaved CX_3CL1 acts as a sensor for neuronal stress, which stimulates microglia to phagocytose excitotoxic neurons (Noda et al., 2011). Given the many facets in which CX_3CL1/CX_3CR1 signaling works to inhibit inflammation and maintain a milieu skewed towards quiescence, it is probable that CX_3CL1 contributes to the preservation of an optimal neurogenic niche for the development, proliferation, and integration of NPCs within the adult CNS.

Taken together, CX_3CL1 plays an important role in maintenance of homeostasis in the adult CNS by mediating neuron-microglia interactions during physiologic conditions. Following CX_3CR1 activation, microglia are known to remove excess neurons and support maturing synapses (Hoshiko et al., 2012; Cunningham et al., 2013; Lenz et al., 2013; Ueno et al., 2013), eliminate apoptotic neural progenitors during adult hippocampal neurogenesis (Sierra et al., 2014), and remodel neuronal circuitry during learning and memory processes (Schafer et al., 2012; Parkhurst et al., 2013). Unmanipulated adult mice deficient in CX_3CR1 had a reduction in "synaptic multiplicity", in which fewer boutons synapsed with more than one postsynaptic spine on a single dendrite. This resulted in reduced connectivity strength between regions of the hippocampus (Zhan et al., 2014), suggesting that CX_3CL1 is required for adult hippocampal plasticity. It is clear that CX_3CL1 is crucial in maintaining homeostasis in the adult CNS; however mechanisms downstream of membrane-bound CX_3CL1- CX_3CR1 binding, as pertains to normal physiology, remain to be fully elucidated.

CONCLUDING REMARKS

While chemokines have historically been thought of as mediators of cell migration, recent evidence suggests that chemokines have the capacity to regulate a number of cellular functions critical to inflammatory processes as well as maintenance of homeostasis. Though the characterization of homeostatic chemokines stems from their roles in lymphoid tissues, many parallels can be drawn in the CNS, particularly in the context of the vasculature. Several chemokines contribute to immune cell trafficking and activation during immunosurveillance (CCL2, CCL19, CCL20, CCL21, CXCL12); regulation of neural progenitor cell migration, proliferation, differentiation, and integration (CXCL12, CXCL14); and maintenance of quiescence (CX_3CL1), orchestrating the balance of homeostasis, while providing immune protection, under physiologic conditions in the CNS. While many functions of chemokines during homeostasis have been identified, there is still much to learn.

Chemokines are known to participate in the function of pericytes within the CNS during physiologic conditions, particularly at the level of the vasculature; however, the role of many of these chemokines is still largely unknown. Pericytes are a key component to the neurovascular unit within the CNS, contributing to endothelial cell tight junction stability and BBB formation (Balabanov and Dore-Duffy, 1998). Song et al. demonstrated that CXCL12 increases pericyte motility *in vitro* and in

a tumor xenograft model *in vivo* (Song et al., 2009), suggesting that pericytes express CXCR4 that facilitates their recruitment to endothelial cells (Virgintino et al., 2013). Vascular pericytes have also been shown to respond to ligands of CXCR3, inducing chemotactic as well as mitogenic effects, stimulating proliferation (Bonacchi et al., 2001). Further, pericytes are known to be a source of chemokine ligands, including CCL3 and CCL4, both constitutively and in response to LPS (Kovac et al., 2011). Due to the spatial distribution of pericytes within the neurovascular unit (**Figure 3**), they are likely to have an important role with regard to maintenance of homeostasis via chemokine regulation. Elucidating the role of chemokines in pericyte function will aid in the understanding of these cells in the context of homeostasis and disease in the CNS.

CCL27 (cutaneous T cell-attracting chemokine, CTACK) is largely expressed by keratinocytes, binds to the receptor CCR10, and may serve as an important regulator of homeostatic immune surveillance. CCL27 has been implicated in inflammatory allergic reactions, primarily in homing memory T cells to the skin (Morales et al., 1999; Huang et al., 2008). Interestingly, Gunsolly et al. have characterized the expression of CCL27 in the cerebral cortex and limbic regions of the CNS in mice. During allergic inflammation induced by intranasal injection of ovalbumin, a variant of CCL27 was up-regulated in the olfactory bulb and was accompanied by infiltration of T cells (Gunsolly et al., 2010), suggesting that CCL27 also has a role in T cell recruitment in the CNS. This pathway may represent a point of access to the CNS tissue for leukocytes that bypasses the BBB, which is lacking at the nasal mucosa, cribriform plate, and perineural spaces of the olfactory bulb (Danielyan et al., 2009). Continued investigation of chemokines in the adult CNS will provide new insights into their functions during physiologic conditions and maintenance of CNS protection, and may identify targets for restoring homeostasis following CNS injury.

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Modulating neurotoxicity through CX3CL1/CX3CR1 signaling

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Since the initial cloning of fractalkine/CX3CL1, it was proposed that the only known member of the CX3C or δ subfamily of chemotactic cytokines could play some significant role in the nervous system, due to its high expression on neurons. The pivotal description of the localization of the unique CX3CL1 receptor, CX3CR1, on microglial cells, firmed up by the generation of *cx3cr1*$^{GFP/GFP}$ mice, opened the road to the hypothesis of some specific key interactions between microglia and neurons mediated by this pair. This expectation has been indeed supported by recent exciting evidence indicating that CX3CL1-mediated microglia-neuron interaction modulates basic physiological activities during development, adulthood and aging, including: synaptic pruning; promoting survival of neurons and neural precursors; modulating synaptic transmission and plasticity; enhancing synapse and network maturation; and facilitating the establishment of neuropathic pain circuits. Beyond playing such fascinating roles in physiological conditions, CX3CL1 signaling has been implicated in different neuropathologies. Early papers demonstrated that the levels of CX3CL1 may be modulated by various toxic stimuli *in vitro* and that CX3CL1 signaling is positively or negatively regulated in EAE and MS, in HIV infection and LPS challenge, in epilepsy, in brain tumors, and in other neuropathologies. In this review we focus on the experimental evidence of CX3CL1 involvement in neuroprotection and survey the common molecular and cellular mechanisms described in different brain diseases.

Keywords: CX3CL1, CX3CR1, microglia, neurotoxicity, signaling

The CX3CL1/CX3CR1 axis, together with CD200/CD200R, have mainly been studied in the context of their involvement in halting potentially toxic activated microglial phenotypes (Biber et al., 2007). The phenotypes embodied by the term "microglia activation" have been hotly debated. Recent nomenclatures proposed M1- and M2-like microglial phenotypes, characterized by the production of pro- or anti-inflammatory cytokines and markers, named after the alternative activation states of macrophages (Mantovani et al., 2005). The transition between these two forms, however, is not all or none and several intermediate microglia phenotypes have been described, together with the identification of overlapping features between alternatively activated phenotypes (Ponomarev et al., 2007, 2013; Olah et al., 2012; Crain et al., 2013). Furthermore, it's now evident that no M1/M2 polarization can be supported, even for peripheral macrophages (Xue et al., 2014).

Given contemporary approaches to genome wide expression profiling, we now face the welcome prospect of devising a robust, meaningful account of microglial reactive phenotypes.

In spite of this complex picture, there is a general consensus on the relatively unique role of the CX3C system in mediating key microglial activities, mainly because of its privileged position at the interface with neurons. Microglia-neuron interaction is dynamic, as revealed by *in vivo* video microscopy using *cx3cr1*$^{GFP/+}$ mice and disclosing that microglia branches continuously survey neuronal surfaces and the cerebral microenvironment in the healthy brain, presumably to sense dysfunctional synapses, damaged neurons, or the presence of potentially dangerous agents (Davalos et al., 2005; Nimmerjahn et al., 2005). Thus, in the adult brain, microglia may exert a sentinel function for neurons and, when neuronal damage occurs, microglia rapidly react to protect or to eliminate neurons, if irreversibly damaged. CX3C signaling is deeply involved in this rescue process, directly modulating different aspects of microglia biology important for neuron protection, like the production of soluble factors directly involved in neuron survival, the modulation of phagocytic activity, but also indirectly affecting other cell types (resident or infiltrating) present in brain parenchyma that, in turn, might influence neuron survival.

CX3CR1 was localized to microglia and CX3CL1 to neurons using *in situ* hybridization, by Harrison et al. (1998). Availability of mice with a GFP fluorescent reporter for CX3CR1 transcription confirmed the former finding in adult mice (Cardona et al., 2006) and subsequent studies showed that CX3CR1 is characteristic of microglia throughout embryogenesis and during the murine lifespan (Ginhoux et al., 2010; Mizutani et al., 2012; Schulz et al., 2012). Subsequently, Kim et al. (2011) developed a CX3CL1 reporter and showed a similar neuronal distribution of CX3CL1 in all regions of the adult brain. These expression patterns are somewhat modulated but do not fundamentally alter in disease models. Therefore, CX3CL1/CX3CR1 signaling provides insight

into microglial–neuronal interactions throughout the lifespan (**Table 1**) and in an immense variety of pathological conditions. This review summarizes a portion of this research.

NEUROTOXICITY MODULATION: CYTOKINE AND GROWTH FACTOR PRODUCTION

The CX3CL1/CX3CR1 signaling participates in the control of production and release of several cytokines from microglia. Earlier *in vitro* studies demonstrated that LPS- and IFNγ-induced release of cytokines such as interleukin-1β (IL-1β), TNFα, 8-isoprostane, NO, and IL-6 in cultured microglia was efficiently blocked by CX3CL1 stimulation (Zujovic et al., 2000, 2001; Mizuno et al., 2003). Since then several papers confirmed and provided further *in vivo* evidence supporting the hypothesis that, together with other molecules like CD200, CD22 and CD172, CX3CL1 signaling in the brain reduces microglia reactivity to toxic stimuli, maintaining microglia in a modulated state (Biber et al., 2007). This hypothesis can be evaluated even though the status of "microglial activation" is profoundly uncertain [see (Biber et al., 2014) for a recent review].

MODULATION OF IL-1β SIGNALING

Interleukin-1β is an inflammatory cytokine playing pivotal roles in local and systemic processes and is a key inducer of peripheral and central immune responses to infection or injury. Inhibition of IL-1β signaling has beneficial effects in a variety of experimental paradigms of acute brain damage. Despite controversial data on its neurotoxic effects *in vivo* and *in vitro*, IL-1β is considered a promising clinical target in several neuropathologies (Allan et al., 2005). In paradigms of brain injury where IL-1β increases, CX3CL1 reduces IL-1β levels, correlating with protection from damage.

Increased production of IL-1β by microglial cells isolated from the brain of *Cx3cr1*$^{-/-}$ mice upon systemic LPS challenge was described by Cardona et al. (2006). *Cx3cr1*$^{-/-}$ microglial cells from LPS-challenged mice provided a toxic insult when transplanted in the forebrain of wild type mice, resulting in neuronal TUNEL labeling. Interestingly these toxic effects were prevented eliminating IL1R signaling either with IL-1 receptor antagonist a (IL-1Ra) or in *IL1*$^{-/-}$ mice.

Cardona et al. (2006) also showed enhanced dopaminergic cell loss in *Cx3cr1*$^{-/-}$ or *Cx3cl1*$^{-/-}$ mice after peripheral MPTP administration. Morganti et al. (2012) asked two follow-on questions: what was the role of cytokine production? and which CX3CL1 isoform (soluble or membrane-associated) was responsible for the neuroprotective effect? They reported that IL-1β signaling was blunted by soluble AAV-CX3CL1, injected in the substantia nigra pars compacts of MPTP-injected *cx3cl1*$^{-/-}$ mice, so that less IL-1β and TNFα were produced in the ventral mesencephalon and microglial CD68 expression was reduced. These alterations in cytokine profile induced by soluble AAV-CX3CL1 correlated with reduced death of dopaminergic TH positive neurons and improved motor coordination on accelerating rotarod. A protective effect of soluble CX3CL1 on dopaminergic neurons was demonstrated by the same authors in a rat model of PD, induced by intrastriatal 6-OHDA injection (Pabon et al., 2011).

CX3CL1 decreased in the brain of aged mice possibly due to neuronal loss, and this change is accompanied by an altered effector state of microglia (Lyons et al., 2009; Wynne et al., 2010; Bachstetter et al., 2011). In aged mice, the response to systemic infection is amplified by the microglia effector response: LPS administration evokes an increased IL-1β and a reduced TGFβ expression, in comparison with young adult animals (Wynne et al., 2010).

Table 1 | Documented effects *in (ex) vivo* of CX3CL1/CX3CR1 signaling in lifespan.

Period	Effects	Mechanisms	Reference
Embryo	Active in early microglial precursors	Unknown	Mizutani et al. (2012)
Postnatal	Circuitry refinement	Synaptic engulfment; timing of microglia	Paolicelli et al. (2011),
	Cortical neuron survival	recruitment	Hoshiko et al. (2012),
	Glutamatergic synapse maturation	IGF-1 production	Zhan et al. (2014)
		Delayed microglia-synapse interaction	Ueno et al. (2013)
		during development	Arnoux et al. (2013)
Adult	Dentate gyrus neurogenesis	IL-1β modulation	Rogers et al. (2011), Maggi et al. (2011),
	Modulation of inflammatory cytokine production	Unknown	Cardona et al. (2006)
	Modulation of glutamatergic neurotransmission	Post-synaptic (GluR1 dephosphorylation; NMDAR potentiation through D-Ser) modulation	Ragozzino et al. (2006), Bertollini et al. (2006), Maggi et al. (2009), Scianni et al. (2013)
	Progenitor cell proliferation in the olfactory bulb	Inflammatory cytokine production and monocyte recruitment	Blomster et al. (2011)
Aging	Dentate gyrus neurogenesis	IL-1β modulation	Bachstetter et al. (2011)
	Microglia effector state	PI-3K	Lyons et al. (2009), Wynne et al. (2010), Bachstetter et al. (2011)

Aging is reported to decrease neurogenesis in the hippocampal region of the brain (Kuhn et al., 1996). Similarly to aging, deficits in CX3CL1 signaling due to gene deletion or caused by CX3CR1 blocking antibodies reduced the number of progenitor cells in the hippocampal dentate gyrus (Bachstetter et al., 2011; Maggi et al., 2011). CX3CL1 administration reverted the age-related decrease in neurogenesis and chronic blockade of CX3CR1 function reduced neurogenesis and increased IL-1β in young adult mice (Bachstetter et al., 2011). IL-1β was implicated in the reduced survival and proliferation rate of neuronal progenitors in the hippocampal region of *CX3CR1*$^{-/-}$ mice, since IL-1Ra reverted the observed phenotypes (Bachstetter et al., 2011; Rogers et al., 2011).

The involvement of IL-1 in CX3CL1-induced effects in experimental models of AD is suggested by several lines of evidence. Mouse deleted of *cx3cr1* showed a strong increase of microtubule-associated protein tau (MAPT) phosphorylation upon LPS challenge, and humanized MAPT transgenic mice crossed with *cx3cr1*$^{-/-}$ mice exhibited accelerated behavioral impairment along with increased MAPT hyperphosphorylation and aggregation. These data, together with the observation that microglia-induced MAPT phosphorylation in neurons can be blocked by IL-1Ra and p38 inhibitors further supports the hypothesis that inhibiting production of IL-1β constitutes a central mechanism for CX3CL1 neuroprotective signaling (Bhaskar et al., 2010). Additional possible targets of CX3CL1 signaling in AD have been suggested: Cho et al. (2011) demonstrated that hAPP/*cx3cr1*$^{-/-}$ mice have exacerbated neuronal and cognitive function defects and increased inflammatory responses in comparison with hAPP/*cx3cr1*$^{+/+}$ mice. The lack of *cx3cr1* in this AD mouse model (hAPP-J20) did not modify plaque deposition but increased tau phosphorylation, cognitive deficits, and microglia activation, along with enhanced IL-6 and TNF-α levels. In line with these observations, AD autopsy brain sections showed reduced CX3CL1 levels in the cortex and hippocampus regions. Further supporting a potential role for CX3CR1 signaling in AD pathogenesis, Lee et al. (2010) reported that in two different mouse models of AD (APPS1 and R1.40), characterized by different velocity of β-amyloid deposition, crossing with *cx3cr1*$^{-/-}$ mice reduced the extent of amyloid deposition, the number of dystrophic neurons and of plaque-associated microglia cells. This protected phenotype was accompanied by increased CCL2 and TNF-α and elevated IL-1β expression. By contrast, a potentially toxic effect for CX3CL1 was suggested by Wu et al. (2013) demonstrating that the deficits induced by β-amyloid injection in hippocampal CA1, as well as the increased IL-1β expression and microglia activation, were all reverted by central CX3CR1 suppression by siRNA delivery. A toxic effect for CX3CR1 signaling in AD was also proposed by Fuhrmann et al. (2010) that found a reduced neuronal loss when the *3xTg AD* mice were crossed with *cx3cr1*$^{-/-}$ mice. In summary, CX3CR1/CX3CL1 signaling modifies AD-related pathology quite consistently but a unified concept of the net outcome of the effects has not been conclusively proven.

Alteration of IL-1β levels was reported in *cx3cr1*-deficient mice upon focal cerebral ischemia. Dénes et al. (2008) demonstrated a reduction in IL-1β and TNF-α production in brains of *cx3cr1*$^{-/-}$ mice upon induction of cerebral ischemia, together with reduced ischemic volume and less neuronal cell death. Similar experiments in a focal brain ischemia model were performed by Soriano et al. (2002) in *cx3cl1* deficient mouse and confirmed lower ischemic volume in the absence of CX3CL1/CX3CR1 signaling. This result was later confirmed in a pMCAO model (Cipriani et al., 2011). In apparent contrast, when CX3CL1 was exogenously administrated to *wt* mice and followed by pMCAO, a significant reduction of ischemic volume was observed while in *cx3cl1*$^{-/-}$ mice CX3CL1 administration worsened the effects of ischemia (Cipriani et al., 2011).The mechanisms underlying divergent effects of CX3CL1 administration to *wt* and *Cx3cl1*$^{-/-}$ mice remain uncertain. In favor of a protective effect of CX3CL1 in cerebral ischemia, Donohue et al. (2012) reported that stroke patients exhibited a positive correlation between high plasma CX3CL1 levels and a better clinical outcome. Interestingly, Pimentel-Coelho et al. (2013) demonstrated a protective role for CX3CR1 in neonatal hypoxic ischemia in female mice, with a possible gender specific protective effect. The authors demonstrated that hippocampal CX3CL1 was reduced by ischemia and that *Cx3cr1*$^{-/-}$ mice showed worse learning deficits and hippocampal damage after ischemia (Pimentel-Coelho et al., 2013).

In an *in vitro* genetic model of amyotrophic lateral sclerosis (ALS), where the mutated SOD1^{G93A} is specifically expressed by astrocytes, Sun et al. (2013) reported that conditioned medium from mesenchymal stem cell (MSC) cultures increased CX3CL1 and reduced TNFα, IL6, and iNOS expression on astrocytes, and mediated a protective effect towards primary motor neurons. *In vivo,* MSC administration improved the functional outcome of mutated mice. In cultured microglia, the presence of mutated SOD1^{G93A} also increased CX3CR1 levels.

Taken together, these data demonstrate that CX3CL1 attenuates inflammatory cytokine production, strongly modulating the neuroprotective activity of microglia in several brain diseases.

PERMISSIVE EFFECTS OF ADENOSINE

Adenosine is a well-established modulator of synaptic transmission and has protective effects in the nervous system, primarily mediated through adenosine type 1 receptor (A_1R). Adenosine is released by neurons and glial cells and its level in the brain is regulated essentially by the activity of adenosine kinase (ADK), and 5′ nucleotidase acting on extracellular ATP (Boison et al., 2010).

Upon CX3CL1 treatment of cultured microglia, increased extracellular adenosine release is observed (Lauro et al., 2008, 2010). In *in vitro* models of excitotoxicity (Lauro et al., 2010) and *in vivo* models of cerebral ischemia, the neuroprotective activity of CX3CL1 requires A_1R activation (Cipriani et al., 2011). This neuroprotective mechanism does not derive from a direct microglia-neuron cross talk, requiring (at least *in vitro*) the involvement of astrocytes whose A_1Rs are stimulated by adenosine released from microglia, increasing astrocyte glutamate transporter (GLT-1) expression and activity (Catalano et al., 2013).

The neuroprotective activity of adenosine has been correlated to its ability to hamper the release of excitatory glutamate at pre-synaptic terminals (Fredholm et al., 1983). In this regard, it is interesting to note that CX3CL1 has a wide spectrum of modulatory activities on glutamatergic neurotransmission, interfering with: (i) pre-synaptic glutamate release, (ii) the amplitude of AMPA currents, thereby altering GluR1 phosphorylation state (post-synaptic modulation), (iii) LTP expression and LTD induction in hippocampal CA1 region, (iv) synaptic NMDAR currents through D-serine release from glia (Meucci et al., 2000; Limatola et al., 2005; Bertollini et al., 2006; Ragozzino et al., 2006; Maggi et al., 2009; Scianni et al., 2013); (v) glutamatergic synapse maturation during development (Paolicelli et al., 2011; Arnoux et al., 2013).

All together these data demonstrate that excitatory neurotransmission, which is deeply implicated in the most common neurotoxic pathways, is affected by CX3CL1/CX3CR1 signaling, with permissive cooperativity of the adenosine system.

GROWTH FACTORS PRODUCTION

Microglia produce a number of growth factors providing trophic support to developing or damaged brain circuits. During brain development, in the first postnatal week, CX3CR1 expression on microglia is important for the survival of cortical neurons of layer V, by regulating the production of insulin-like growth factor-1 (IGF-1; Ueno et al., 2013). In particular, increased death of layer V neurons was observed in the cerebral cortex of *cx3cr1*$^{GFP/GFP}$ mice, in which microglia produced a significantly reduced amount of the growth factor IGF-1. So during brain development, CX3CR1-mediated IGF-1 secretion from microglia modulates neuronal survival in postnatal cortical layer V.

A specific BDNF polymorphism (Val66Met) is associated with memory deficits and increased vulnerability to anxiety and depressive disorders both in humans and mice. Interestingly, this polymorphism also associates with impaired CX3CL1/CX3CR1 hippocampal signaling, reducing expression of CX3CL1 protein and mRNA in dorsal hippocampus (Wang et al., 2014). Chronic CX3CL1 infusion in the hippocampal region of *Val66Met* mice recovered memory deficits, restored neurogenesis in the DG and increased Akt phosphorylation levels.

NEUROTOXICITY MODULATION: MICROGLIA PHAGOCYTIC ACTIVITY

One of the most intriguing functions of microglia in the central nervous system is certainly its activity as the resident phagocyte. In development and in pathological neuroinflammatory conditions, microglial phagocytosis is important for the elimination of dead and damaged neurons, myelin residues and β-amyloid peptides [reviewed in (Sierra et al., 2013)]. During normal brain development, microglial phagocytosis is involved in eliminating supernumerary neurons, but also in synaptic pruning and circuit refinement (Sierra et al., 2010; Tremblay et al., 2010). Among the key factors that emerged as potential modulators of neuron phagocytosis by microglia during development is the complement system, with the proteins C1q and C3 expressed on synapses and microglial CR3/CD11b mediating the elimination of immature and weakly active synapses (Schafer et al., 2012). TGF-β produced by astrocytes signals to retinal ganglion cells for C1q expression (Bialas and Stevens, 2013), thus favoring retinogeniculate refining, and inserting astrocytes in microglia-neuron communication.

Cell damage causes high-grade ATP release (both from damaged cells and through astrocyte exocytosis) regulating microglial process extension (Davalos et al., 2005) and also contributing to inflammasome activation within microglia (Ransohoff and Brown, 2012). Under stress, neurons manifest the phosphatidylserine (PS) "eat me" signal which can be recognized by several microglia receptor/adaptor complexes including Gas6/MerTK and MFGE8/vitronectin receptor (De Simone et al., 2004; Elliott et al., 2009). In ischemic conditions potentially viable neurons reversibly express PS on their surface and are vulnerable to be taken up and destroyed by microglia (Neher et al., 2011). Selective elimination of MFGE8/vitronectin receptor "eat me" signaling strongly improved experimental stroke outcome (Neher et al., 2013).

CX3CR1 signaling also modulates microglia phagocytosis of neurons and their processes in both physiological and pathological conditions. During development, CX3CL1/CX3CR1 signaling contributes to refinement of synaptic elements in varied CNS regions during the postnatal "critical period" (Tremblay et al., 2010; Paolicelli et al., 2011; Hoshiko et al., 2012). This fine-tuning of anatomical connections is important to establish and build an optimal functional circuitry (Zhan et al., 2014).

In Alzheimer's disease (AD), CX3CL1/CX3CR1 blockade modulates microglia phagocytic activity in a way that markedly attenuates amyloid deposition (Lee et al., 2010; Liu et al., 2010). In particular, CX3CR1 deficient microglia show increased phagocytic activity when crossed with different AD mouse models CRND8 (Liu et al., 2010) or APPPS1 and R1.40 (Lee et al., 2010), resulting in attenuated Aβ deposition. Based on results that look beyond amyloid deposition in various models, the overall outcome of eliminating CX3CR1 signaling remains in doubt (Bhaskar et al., 2010; Fuhrmann et al., 2010; Cho et al., 2011).

Neurons damaged by toxic insults, like glutamate, release more CX3CL1 that increases MFG-E8 expression on microglia (Leonardi-Essmann et al., 2005; Fuller and Van Eldik, 2008; Noda et al., 2011). As noted above, MFG-E8 is a PS receptor that recognize PS on injured neurons and acts as an adaptor recognized by microglial vitronectin receptor, inducing damaged-cell uptake. CX3CL1 up-regulation of MFG-E8 expression was associated to increased heme oxygenase 1 (HO-1) expression through the MAPKs ERK and JNK and the nuclear factor erythroid 2 related factor (NFE2LE). Lastres-Becker et al. (2014) reported that CX3CL1 reduced Tau-induced microgliosis via up-regulating transcription factor NRF2/NFE2LE, along with increased HO-1 expression. Since the increased expression of HO-1 is reported to mediate anti inflammatory activities (Chora et al., 2007), this pathway could be involved in the reported neuroprotective effects of CX3CL1 in different Tau pathology models (Nash et al., 2013).

These data indicate that the clearance and phagocytic activity of microglia can be modulated by CX3CL1 signaling through

varied mechanisms, and that the final outcome in term of neuroprotection or neurotoxicity might result from the presence of co-stimulatory signals and, consequently, from the effector programs of microglia.

NEUROTOXICITY MODULATION: EFFECTS ON NEURAL PRECURSORS

In vitro, CX3CL1 promoted survival of neural precursor cells upon growth factor withdrawal from the culture medium (Krathwohl and Kaiser, 2004). Further, *cx3cr1*$^{-/-}$ mice have reduced neurogenesis in the hippocampal DG region (Bachstetter et al., 2011; Maggi et al., 2011) but this reduction was accompanied by contrasting effects on memory deficits and synaptic plasticity: Maggi et al., (Maggi et al., 2011) reported that *cx3cr1*$^{-/-}$ mice had increased hippocampal LTP and performed better in the Morris water maze test, while, in the same mouse strain, Rogers et al. (2011) described reduced hippocampal LTP and deficits in the Morris water maze. At least in part, these conflicting data reflect the lack of simple relations between DG neurogenesis, plasticity processes in the hippocampal CA1 region and learning behavior.

More recently, Vukovic et al. (2012) demonstrated that CX3CR1 deficiency impairs neurogenesis both in young and aged mice and that voluntary physical exercise increased brain CX3CL1 levels and DG neurogenesis, both effects being abolished by intrahippocampal CX3CR1 antibody injection.

In the olfactory bulb, CX3CR1 deletion increased olfactory sensory neuron death upon bulbectomy and *cx3cr1*$^{-/-}$ mice showed reduced proliferation of intraepithelial stem progenitor cells, increased macrophage recruitment in the lesioned epithelium and increased TNF-α and IL-6 levels (Blomster et al., 2011).

All together these data suggest that neuroprotective effects of CX3CL1 might arise partly from an increased proliferation or survival of progenitor elements in selected brain regions where neurogenesis continues throughout adult life.

NEUROTOXICITY MODULATION: INDIRECT EFFECTS

MODULATION OF ASTROCYTE ACTIVITY

In the CNS, the unique expression of CX3CR1 on microglia clearly suggests that the neuroprotective effects of CX3CL1 depend primarily on the direct communication between microglia and neurons. Recent evidence suggested that CX3CL1/CX3CR1 signaling in neuroprotection again excitotoxic insult might not be restricted to a direct microglia-neuron communication but also involves indirectly modulation of astrocyte activity. Catalano et al. (2013), in fact, demonstrated that CX3CL1, acting on microglia, induced the production and release of soluble factors that exerted their effects on astrocytes, inducing the functional up regulation and the increased expression of the excitatory amino acid transporter GLT-1. As already reported in *in vitro* and *in vivo* systems (Lauro et al., 2010; Cipriani et al., 2011), this cross-talk requires the "permissive" presence of adenosine, specifically acting on astrocyte A_1R (Catalano et al., 2013). These data demonstrated for the first time a role for astrocytes in mediating the neuroprotection induced by CX3CL1/CX3CR1 signaling between microglia and neurons.

EFFECTS THROUGH LEUKOCYTE RECRUITMENT

Neuroprotective effects of CX3CL1 could also arise from hematogenous leukocytes that enter the brain upon specific, injury-induced challenge.

In mouse experimental allergic encephalomyelitis (EAE) Huang et al. (2006) demonstrated that CX3CR1 deficient mice had an exacerbated phenotype, with severe spastic paralysis and hemorrhagic inflammation in the CNS, resulting in higher mortality. The increased toxicity resulted from an altered control of NK cells entry into the brain of EAE-affected animals. These findings were extended by Garcia et al. (2013), who showed that CX3CR1 deficiency limited to bone marrow cells of mice with EAE resulted in more severe pathology, with increased demyelination, axonal damage and reduced calbindin positive neurons in the cerebellum. These effects correlated with increased CD115^{+}, Ly6C^{low}CD11c^{+} dendritic cell infiltration in the CNS and increased IL-17 and IFNγ expression in the cerebellum, forebrain and spinal cord.

Mills et al. (2008) reported that in *CD73*$^{-/-}$ mice, where the ectonucleotidase responsible for extracellular adenosine accumulation was absent, EAE induction produced a milder phenotype. More recently, the same group (Mills et al., 2012) reported that extracellular adenosine increased CX3CL1 expression and that CX3CL1 promoted lymphocyte entry into the brain of EAE mice. Therefore, in CD73 deficient mice, extracellular adenosine cannot be produced, CX3CL1 is not upregulated and a reduced amount of lymphocytes enter cerebral parenchyma, resulting in a protected phenotype (Mills et al., 2012).

In focal cerebral ischemia, Dénes et al. (2008) reported a reduced leukocyte (CD45^{+} cells) infiltration in the brain of *cx3cr1*$^{-/-}$ mice and a consistently reduced damaging of the blood brain barrier. This correlated with a protected phenotype, with reduced ischemic volume and lesser numbers of apoptotic cells and a better performance in the behavioral test of adhesive tape removal.

All together these data highlight that CX3CL1/CX3CR1 signaling modulates the entry of hematogenous leukocytes, with beneficial or deleterious consequences contingent on context.

CONCLUSION

This review highlights the manifold effects of altering CX3CR1/CX3CL1 signaling, which can be observed throughout the lifespan from early embryogenesis through diseases of the aging brain. For the most part, CX3CR1/CX3CL1 can be viewed as a fulcrum with which to modify microglial physiology to probe into the panoply of functions of these versatile and essential cells. Adding to complexity but also lending heuristic value, the effects of modifying CX3CR1/CX3CL1 pathways are extremely context dependent. Nevertheless in each case insights about upstream and downstream signaling from CX3CR1/CX3CL1 have been informative and may point to new therapeutic directions for brain disease. Finally, it is now clear that macroglia (such as astrocytes) and hematogenous leukocytes also contribute to the varied physiology of the CX3CR1/CX3CL1 signaling system.

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Trasmembrane chemokines CX3CL1 and CXCL16 drive interplay between neurons, microglia and astrocytes to counteract pMCAO and excitotoxic neuronal death

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Upon noxious insults, cells of the brain parenchyma activate endogenous self-protective mechanisms to counteract brain damage. Interplay between microglia and astrocytes can be determinant to build a physiological response to noxious stimuli arisen from injury or stress, thus understanding the cross talk between microglia and astrocytes would be helpful to elucidate the role of glial cells in endogenous protective mechanisms and might contribute to the development of new strategy to mobilize such program and reduce brain cell death. Here we demonstrate that chemokines CX3CL1 and CXCL16 are molecular players that synergistically drive cross-talk between neurons, microglia and astrocytes to promote physiological neuroprotective mechanisms that counteract neuronal cell death due to ischemic and excitotoxic insults. In an *in vivo* model of permanent middle cerebral artery occlusion (pMCAO) we found that exogenous administration of soluble CXCL16 reduces ischemic volume and that, upon pMCAO, endogenous CXCL16 signaling restrains brain damage, being ischemic volume reduced in mice that lack CXCL16 receptor. We demonstrated that CX3CL1, acting on microglia, elicits CXCL16 release from glia and this is important to induce neroprotection since lack of CXCL16 signaling impairs CX3CL1 neuroprotection against both *in vitro* Glu-excitotoxic insult and pMCAO. Moreover the activity of adenosine receptor A3R and the astrocytic release of CCL2 play also a role in trasmembrane chemokine neuroprotective effect, since their inactivation reduces CX3CL1- and CXCL16 induced neuroprotection.

Keywords: CX3CL1, CXCL16, CCL2, A3R, glia cross-talk, neuroprotection, ischemia, excitotoxicity

INTRODUCTION

Glial cells, able to sense changes in brain environment, represent active players in various pathological conditions such as chronic neurodegenerative disease, trauma and stroke. It is now established that both microglia and astrocytes can play dual roles in the CNS having either detrimental or beneficial effects participating and enhancing inflammatory conditions, or limiting neuroinflammation, favoring repair and enhancing neuronal survival (Liu et al., 2011). Thus understanding the cross talk between microglia and astrocytes would be helpful to elucidate the role of glial cells in pathological conditions.

Microglia-astrocytes interplay is granted by different types of soluble mediators including ATP, adenosine, glutamate (Glu) (Boison et al., 2010; Burnstock et al., 2011; Franke et al., 2012; Pascual et al., 2012), growth factors and inflammatory cytokines (Hamby and Sofroniew, 2010). We have recently shown that the transmembrane chemokine CXCL16 and its receptor, CXCR6, are constitutively expressed in glia and neurons being able to drive neuroprotection against Glu excitotoxicity and oxygen glucose deprivation (OGD) insults in culture (Rosito et al., 2012). In particular we found that the neuroprotective activity of CXCL16 involves astrocytic release of CCL2 and the synergistic activity of adenosine and adenosine type 3 receptor (A3R) on astrocytes.

The other known trasmembrane chemokine CX3CL1 is constitutively expressed in the brain only by neurons, while its unique receptor CX3CR1 is exclusively present on microglial cells. Recently described as a neuronal "off signal" that keep microglia in resting state (Biber et al., 2007), in the last decade the role of CX3CL1-CX3CR1 signaling in modulating neuron viability has emerged in several studies on neurodegenerative and neuroinflammatory disease models (Soriano et al., 2002; Cardona et al., 2006; Huang et al., 2006; Dénes et al., 2008; Bhaskar et al., 2010; Fuhrmann et al., 2010; Lee et al., 2010; Cipriani et al., 2011). Moreover CX3CL1 ability to preserve neurons from excitotoxic insult has been shown both *in vitro* and *in vivo*: in particular CX3CL1 signaling in microglia determines the release of soluble factors, such as adenosine that, acting on the adenosine receptor type 1 (A1R), concur to neuroprotection against Glu

excitotoxicity and cerebral ischemia (Limatola et al., 2005; Lauro et al., 2010; Cipriani et al., 2011; Catalano et al., 2013).

In the present paper we studied the interplay between trasmembrane chemokines and between glial cells in determining neuroprotection against excitotoxic insults. In particular we found that: (i) CXCL16 is able to reduce ischemic brain volume; (ii) following ischemic insults there is an overexpression of CXCL16; (iii) CXCL16 and CCL2 are released from glia upon CX3CL1 stimulation; and (iv) A3R and CXCR6 concur to CX3CL1 mediated neuroprotection.

MATERIALS AND METHODS

ANIMALS

Procedures using laboratory animals were in accordance with the international guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609/EEC). C57BL/6J (*wt*) and homozygous cxcr6$^{gfp/gfp}$ knock-in mice (Unutmaz et al., 2000) in which the coding region of the receptor has been substituted with the coding region of the Green Fluorescent Protein (GFP) were obtained from Jackson Laboratory (strain name B6.129P2-Cxcr6tm1Litt/J). A3R knockout mice (A3R$^{-/-}$) (Salvatore et al., 2000) were also used. Animals of either sex were used.

PERMANENT MIDDLE CEREBRAL ARTERY OCCLUSION (pMCAO)

Male mice (25–28 g, 10–12 weeks) were anesthetized with intraperitoneal Equitensine at 3.5 ml/kg (39 mM pentobarbital, 256 mM chloral hydrate, 86 mM MgSO4, 10% ethanol v/v, and 39.6% propyleneglycol v/v). The right MCA was permanently occluded by electrocoagulation as described previously (Storini et al., 2006). Mice were maintained at 37°C during surgery and sacrificed 24 h after pMCAO.

INTRACEREBROVENTRICUL (I.C.V.) INJECTION

Recombinant mouse CXCL16 or mouse CX3CL1 (Peprotech) was dissolved in saline solution and intracerebroventricularly injected 30 min before pMCAO. For dose-response experiments, mice were injected with 15, 70 and 150 pmol CXCL16/2 μl. Anesthetized animals were immobilized on a stereotaxic apparatus (David Kopf Instruments) and injected in the right cerebral ventricle (1 mm lateral and 3 mm deep, according to the atlas of Paxinos and Franklin, 2004). A constant rate of infusion (0.2 μl/min) was maintained with a pump (KD Scientific).

BRAIN ISCHEMIC VOLUME MEASUREMENT

The extent of ischemic area was evaluated 24 h after ischemia. Mice were deeply anesthetized with Equitensine and transcardially perfused with ice-cold PBS (20 ml), pH 7.4, and paraformaldehyde (PFA; 4%, 50 ml) in PBS. The brains were carefully removed from the skull and transferred in 4% PFA at 4°C overnight, then to PBS/30% sucrose at 4°C overnight, frozen in isopentane at −45°C for 3 min, and then stored at −80°C until use. Twenty μm coronal brain cryosections were cut serially at 320 μm intervals and stained with cresyl violet. Infarct volumes were calculated by integration of the infarct areas on each brain slice, as described previously (Storini et al., 2006).

PRIMARY HIPPOCAMPAL CULTURES

Primary hippocampal cultures were prepared from the brain of 0–2-day-old wild type (*wt*), cxcr6$^{gfp/gfp}$ and A3R$^{-/-}$ mice. In brief, after careful dissection from diencephalic structures, the meninges were removed and the hippocampi chopped and digested in 0.025% trypsin, in Hank's balanced salt solution (HBSS) for 20 min at 37°C. Cells were mechanically dissociated and plated at a density of 2.5×10^5 in poly-L-lysine coated plastic 24-well dishes, in serum-free Neurobasal medium supplemented with B27, 0.5 mM L-glutamine and 100 μg/ml gentamicin. Successively, cells were kept at 37°C in 5% CO_2 for 10–11 days *in vitro* (DIV) with a twice a week medium replacement (1:1 ratio). With this method we obtained 60–70% neurons, 30–35% astrocytes, 4–5% microglia, as determined with β-tubulin III, glial fibrillary acidic protein (GFAP), and isolectin IB4 staining (Lauro et al., 2010).

OXYGEN GLUCOSE DEPRIVATION (OGD)

Primary hippocampal cultures (10–11 DIV) were exposed to OGD. Briefly, culture medium was replaced with modified Locke's buffer (without glucose), bubbled with 95% N_2/5% CO_2, and transferred into an anaerobic chamber (Billups-Rothenberg MIC-101) containing a mixture of 95% N_2/5% CO_2, and humidified at 37°C for 90 min. For the reperfusion conditions OGD was terminated by replacing the OGD medium with the original conditioned medium. For comparative purposes, control cultures were treated under normoxic conditions (95% O_2/5% CO_2) in complete Locke's buffer supplemented with glucose (5.6 mM).

GLU EXCITOTOXICITY

In primary hippocampal cultures (10–11 DIV) conditioned medium was removed and stored for later usage; neurons were washed and stimulated with Glu (100 μM, 30 min) in modified Locke's buffer (without $MgCl_2$ plus 1 μM glycine to stimulate all types of Glu receptors), in the presence or in the absence of recombinant mouse mCX3CL1 (100 nM, Peprotech). Under these experimental conditions, only neurons die (Chen et al., 2000; Rosito et al., 2012). After treatment, cells were re-incubated in the original conditioned medium for 18–20 h, treated with lysis buffer (0.5% ethylhexadecyldimethylammonium bromide, 0.28% acetic acid, 0.5% Triton X-100, 3 mM NaC1, 2 mM MgCl, in PBS pH 7.4) and counted in a hemocytometer for viability, as described (Volontè et al., 1994). Data were expressed as percentage of viable cells taking as 100% the number of viable cells in control cultures. Variability in the number of viable cells in control conditions never exceeded 10%.When necessary, cells were pretreated with monoclonal mouse αCCL2 Ab (3 μg/ml, 30 min; R&D MAB479), rat IgG (3 μg/ml, 30 min; Santa Cruz Biotecnology sc-2032), 3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2phenyl-4-propyl-3-pyrinide carboxylate (MRS1523; 100 nM, Sigma) in culture medium; drugs were present also during and after Glu challenge.

RNA EXTRACTION AND ANALYSIS

Total RNA from ipsilateral (ischemic core and penumbra) and controlateral (corresponding areas) brain emispheres of pMCAO

mice, from primary hippocampal mixed cell cultures (5×10^5 cells), from primary astrocytes (2.5×10^5) and microglial cells (2.5×10^5), was extracted by the use of Trizol reagent (Invitrogen). Reverse transcription reaction was performed in a thermocycler (MJ Mini Personal Thermal Cycler; Biorad) using IScriptTM Reverse Transcription Supermix (Biorad) according to the manufacturer's protocol. Real-time PCR (RT-PCR) was carried out in a I-Cycler IQ Multicolor RT-PCR Detection System (Biorad) using SsoFast EvaGreen Supermix (Biorad) according to the manufacturer's instructions. The PCR protocol consisted of 40 cycles of denaturation at 95°C for 30 s and annealing/extension at 58°C for 30 s. For quantification analysis the comparative Threshold Cycle (Ct) method was used. The Ct values from each gene were normalized to the Ct value of β-actin or GAPDH in the same RNA samples. Relative quantification was performed using the $2-\Delta\Delta$Ct method (Schmittgen and Livak, 2008) and expressed as fold change in arbitrary values. Primer sequences targeted against CXCL16 (BC019961.1, GenBank), mouse β-actin and GAPDH were as follows: CXCL16-forw. TCCTTTTCTTGTTGGCGCTG, CXCL16rev. CAGCGACACT-GCCCCTGGT; β-actin-forw.AGAGGGAAATCGTGCGTGAC, β-actin-rev. CAATAGTGATGACCTGGCCGT; GAPDH-forw. TCGTCCCGTAGACAAAATGG, GAPDH-rev. CAAGGGGTTGAAGCTCAGAT.

GLIAL PRIMARY CULTURES

Primary cortical glial cells were prepared from 0–2-day-old *wt* mice. Cerebral cortices were chopped and digested in 30 U/ml papain for 40 min at 37°C followed by gentle trituration. The dissociated cells were washed, suspended in DMEM with 10% fetal bovine serum (FBS; Gibco) and 2 mM L-glutamine and plated at a density of $9–10 \times 10^5$ in 175 cm^2 cell culture flasks. At confluence (10–14 DIV), glial cells were shaken for 2 h at 37°C to detach and collect microglial cells. Astrocytes which remained attached to the bottom of the flask were treated with trypsin and collected. These procedures gave almost pure (no more than 2% astrocyte contamination) microglial cell population, and astrocytes cell population (4–6% of microglia contamination), as verified by staining with GFAP and isolectin IB4.

MICROGLIA-ASTROCYTE CO-CULTURES

After 10–14 DIV, 8×10^5 microglial cells were re-plated and co-cultured for 48 h with astrocytes cells (8×10^5) seeded on 24 mm transwell cell-culture inserts (pore size 0.4 μm; Corning Life Sciences) which allows traffic of small diffusible substances, but prevents cell contact. After co-cultures, cells were treated with vehicle or soluble CX3CL1 (100 nM) for 18 h and upon stimulation proteins from cells and conditioned medium was collected and analyzed for Western blot. For CCL2 ELISA and mRNA analysis 2.5×10^5 astrocyte were re-plated on 12 mm transwell cell-culture inserts (pore size 0.4 μm; Corning Life Sciences) and co-cultured with 2.5×10^5 microglial cells. After 48 h cells were treated with vehicle or soluble CX3CL1 (100 nM) for 18 h. For ELISA conditioned medium (c.m.) was collected and analyzed according to the manufacturer (R&D Systems), while for mRNA extraction microglia and astrocytes were collected from the different transwell compartments.

PROTEINS PREPARATION

C.m. from microglia-astrocytes co-cultures were collected and concentrated by ultrafiltering on Ym-10 membrane (Centricon; Millipore). For cell membrane proteins preparation, cells were washed with phosphate-buffered saline and lysed for 15 min on ice in hypotonic buffer containing 10 mM HEPES pH 8, 1.5 mM $MgCl_2$, 1 mM DDT, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 1500 rpm for 5 min at 4°C, supernatant were ultra-centrifuged at 55000 rpm for 60 min, 4°C, and pellet were suspended in NaCl 10 mM.

WESTERN BLOT ANALYSIS

Protein samples were separated on 10% SDS-polyacrylamide gel and analyzed by Western immunoblot using a mouse CXCL16 antibody (0.2 μg/ml; R&D System, AF503) and HRP-tagged rabbit anti goat IgG secondary antibody (1:2000; Dako), and subsequently detected using a commercial chemiluminescent assay (Immun-Star WesternC Kit; Bio-Rad). Densitometric analysis was performed with Quantity One software (Biorad). Interpretation of western-blot bands for CXCL16 was according to Gough et al. (2004).

STATISTICAL ANALYSIS

The data are expressed as the means ± SEM. Where appropriate *t*-test, or analysis of variance (ANOVA) was used: we performed the parametric one-way ANOVA or two-way ANOVA followed by specific multiple comparison, as described in detail in figure legends. A value of $p < 0.05$ was considered significant. All statistical analysis was done using SigmaPlot 11.0 Software.

RESULTS

SOLUBLE CXCL16 REDUCES THE ISCHEMIC VOLUME IN MOUSE BRAIN AFTER pMCAO

Since we have recently demonstrated that CXCL16 is neuroprotective against Glu excitotoxicity *in vitro* (Rosito et al., 2012), we now investigated the ability of CXCL16 to induce neuroprotection in mice upon pMCAO. In *wt* mice, i.c.v. injection of soluble CXCL16, 30 min before induction of pMCAO, resulted in a decreased ischemic volume compared to *wt* mice injected with saline ($n = 10$) control solution: in particular, a significant reduction in ischemic volume was observed after injection of 70 and 150 pmol of CXCL16 ($n = 4–8$; $p < 0.05$), while no effect was observed upon injection of 15 pmol ($n = 4$; **Figure 1A**). Further experiments were performed at 70 pmol. The neuroprotective effect of CXCL16 was specific, being absent in mice that lack CXCR6 receptor ($cxcr6^{gfp/gfp}$ mice; **Figure 1B**). Two-way ANOVA analysis indicated a significant interaction between genotypes and treatments ($p = 0.02$) and *post hoc* evaluation revealed that CXCL16 was ineffective in reducing ischemic volume in $cxcr6^{gfp/gfp}$ mice ($n = 6$). In addition, in $cxcr6^{gfp/gfp}$ mice, pMCAO induced a significantly increased ischemic volume compared to *wt* animals suggesting that endogenous CXCL16-CXCR6 signaling contributes to restrain brain damage following ischemic insult.

To investigate whether the protective effect of CXCL16 upon pMCAO requires the activity of A3R, we analyzed the effect

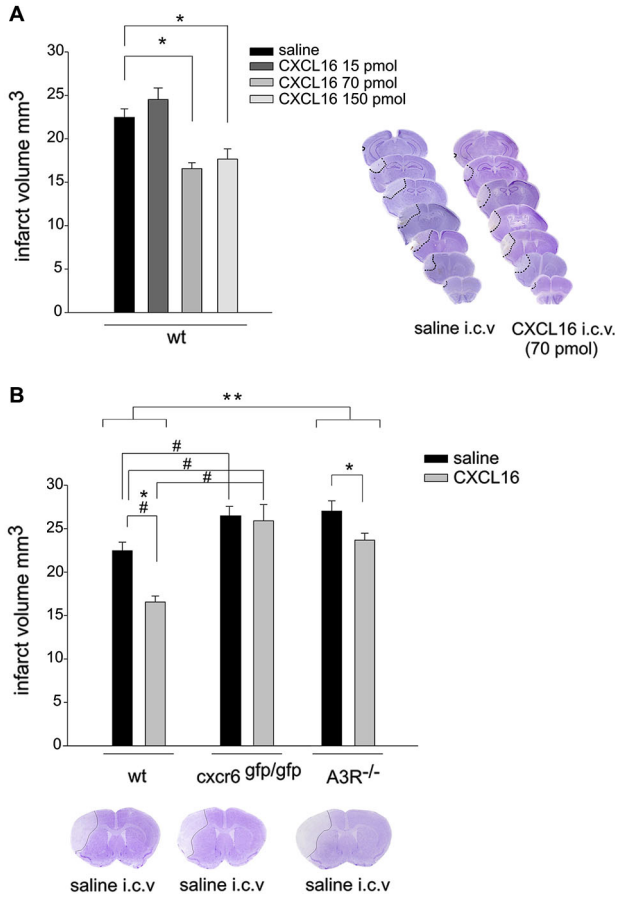

FIGURE 1 | CXCL16 reduces ischemic volume in pMCAO. (A) CXCL16 dose-response effect. Left: Mice were i.c.v. injected with saline or CXCL16 (15–70-150 pmol) 30 min before pMCAO and analyzed for ischemic volume 24 h later (n = 4–10). Right: representative brain coronal sections from saline and CXCL16 i.c.v injected mice. Pale demarcated areas depict the ischemic lesions. **(B)** CXCL16 effect in *wt*, cxcr6 $^{gfp/gfp}$, and A3R$^{-/-}$ mice. Mice of different genotypes (as indicated) were injected with saline or CXCL16 (70 pmol) before pMCAO and analyzed for ischemic volume (n = 6–10). Representative brain coronal section of pMCAO saline injected mice of different genotypes (Bottom). Results represent the mean ± SEM. Statistical analysis: one-way ANOVA followed by Holm-Sidak *post-hoc* test *$p < 0.05$ **(A)**. Two-way ANOVA followed by Holm-Sidak *post-hoc* test * $p < 0.05$; ** $p < 0.001$; * refers to *wt*-A3R$^{-/-}$comparison; $^{\#}$ $p < 0.05$ refers to *wt*-cxcr6$^{gfp/gfp}$ comparison **(B)**.

of i.c.v. administration of CXCL16 in A3R$^{-/-}$ mice (n = 6–7; **Figure 1B**). Two-way ANOVA analysis reveals a significant differences between genotypes ($p < 0.001$), being the ischemic volume higher in A3R$^{-/-}$ vs. *wt* animals. CXCL16 administration was effective in reducing ischemic volume in both genotypes, ($p < 0.05$) but the reduction observed in A3R$^{-/-}$ mice was less pronounced (12.3% in A3R$^{-/-}$ vs. 26.3 % in *wt*).

ISCHEMIC INSULTS INDUCE UP-REGULATION OF ENDOGENOUS CXCL16

Since CXCL16 signaling is determinant in reducing brain damage, we measured CXCL16 expression in the brain upon ischemia. RT-PCR analysis revealed that 24 h after pMCAO CXCL16 mRNA specifically increased in the ipsilateral hemisphere (n = 5; $p <$ 0.001; **Figure 2A**), while no differences were observed in sham operated mice (not shown). Similar results were obtained *in vitro*, when hippocampal cultures were treated to induce OGD cell death (Rosito et al., 2012). After 90 min of OGD we observed a reduction of CXCL16 mRNA, followed by a significant increase after 2 h of recovery (n = 8–11; $p < 0.05$; **Figure 2B**).

CXCL16 IS RELEASED FROM MICROGLIA AND ASTROCYTES UPON CX3CL1 STIMULATION

To investigate if CXCL16 could be released from glia upon treatment with the neuroprotective chemokine CX3CL1, conditioned media (c.m.) from microglia/astrocytes co-cultures (in transwell system, see Section Materials and Methods) treated or not with CX3CL1 (100 nM, 18 h), were analyzed for CXCL16 presence. Data shown in **Figure 3A** revealed a significant increase in soluble CXCL16 upon CX3CL1 treatment (n = 12; $p < 0.001$). Membrane fractions of both microglia and astrocytes were also analyzed and we found that after CX3CL1 treatment the mature form of CXCL16 was significantly increased (**Figures 3B,C** top panels; n = 6; $p < 0.05$). Interestingly, we also observed an increased expression of CXCL16 mRNA upon CX3CL1 stimulation both in microglia and astrocytes (**Figures 3B,C** bottom panels; n= 6–8; $p < 0.05$).

CXCL16 IS A MEDIATOR OF CX3CL1-INDUCED NEUROPROTECTION AGAINST GLU EXCITOTOXICITY

Since CXCL16 acts on its unique receptor CXCR6, we performed experiments on the neuroprotective activity of CX3CL1 against Glu excitotoxicity in hippocampal cultures obtained from cxcr6$^{gfp/gfp}$ mice, to investigate the possible involvement of CXCL16 in CX3CL1-induced neuroprotection. As reported in **Figure 4**, CX3CL1 was less effective in preventing cell death in hippocampal cultures derived from mice that lack CXCL16 signaling, compared with *wt* cultures. In particular, statistical analysis (two-way ANOVA) indicated a significant interaction between genotypes and treatment, with a main effect of treatments (p = 0.004). In cxcr6$^{gfp/gfp}$ mice, a significant difference between Glu and both control and Glu/CX3CL1 treated cells was observed (n = 8–10; $p < 0.05$).

THE MEDIATORS OF CXCL16 NEUROPROTECTIVE ACTIVITY ARE ACTIVE PLAYERS IN CX3CL1 NEUROPROTECTION

The activity of CXCL16 and A3R on astrocytes and the consequential release of CCL2 are key events in CXCL16 induced neuroprotection (Rosito et al., 2012). To further corroborate the CX3CL1-CXCL16 connection in neuroprotection, we analyzed the contribution of A3R and CCL2 in this mechanism. We performed excitotoxic experiments in hippocampal cultures derived from A3R$^{-/-}$ mice: at difference with *wt*, in A3R$^{-/-}$ cultures CX3CL1 was less effective in preventing cell death (**Figure 5A**). Statistical analysis (two-way ANOVA) indicated a significant interaction between genotypes and treatment (p = 0.025), with a main effect of treatments. Both in *wt* and A3R$^{-/-}$ animals, a significant difference between Glu and both control and Glu/CX3CL1 treated cells was observed (*post hoc* analysis,

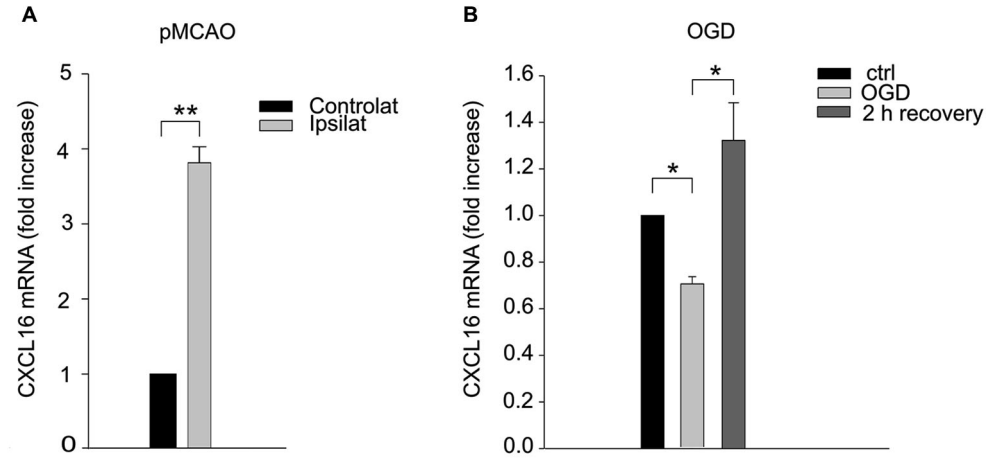

FIGURE 2 | CXCL16 expression is increased upon ischemic insults. (A) CXCL16 mRNA quantification in pMCAO brain. qRT-PCR analysis in contralateral and ipsilateral emisphere 24 h after pMCAO ($n = 5$). **(B)** CXCL16 mRNA quantification in *in vitro* OGD. qRT-PCR analysis in primary hippocampal cultures after 90 min of OGD ($n = 11$) and after 2 h of recovery ($n = 8$). Data are expressed as mRNA fold increase normalized to β-actin. Results represent the mean ± SEM. Statistical analysis: Student's *t*-test ** $p < 0.001$ **(A)**; one-way ANOVA followed by Dunn's *post-hoc* test * $p < 0.05$ **(B)**.

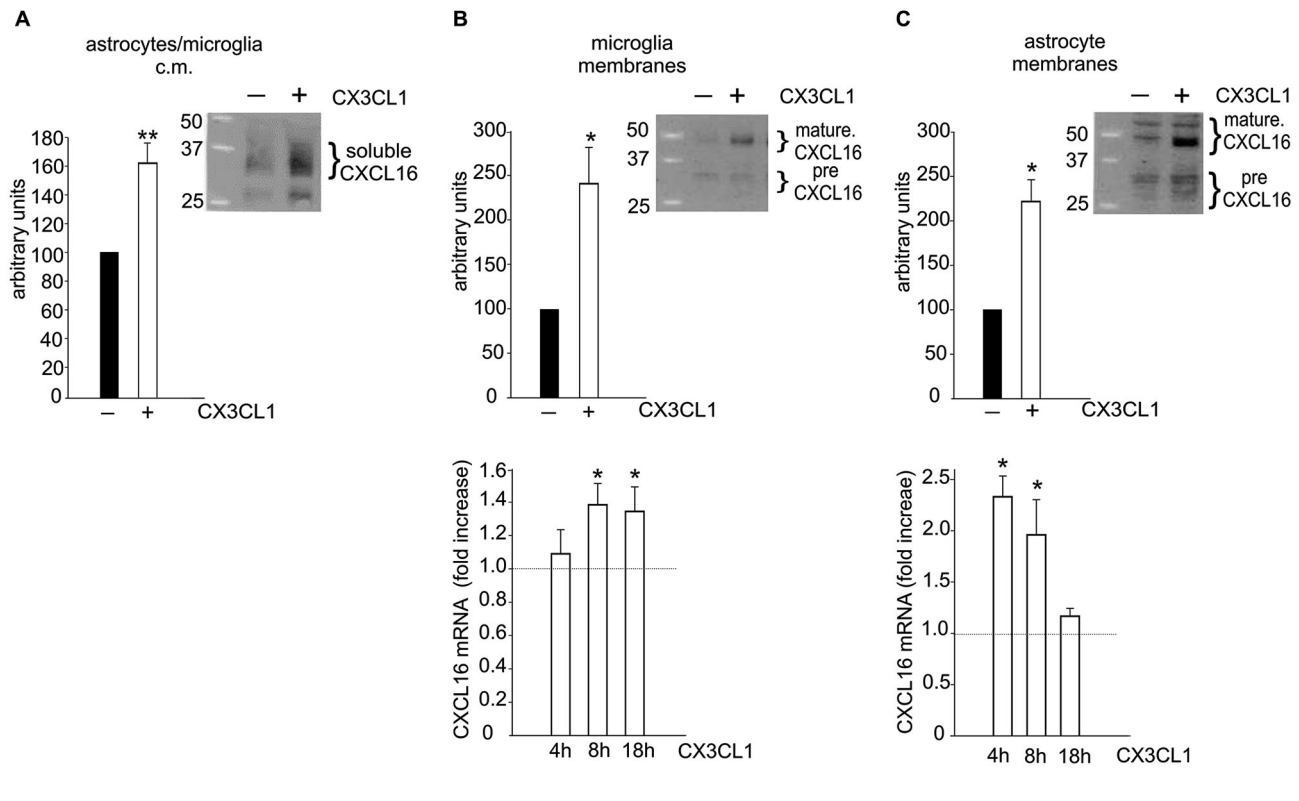

FIGURE 3 | Glial cells increase CXCL16 expression upon CX3CL1 stimulation. (A) CX3CL1 induces the release of soluble CXCL16 from glial cells. Western-blot analysis for CXCL16 protein in the conditioned medium (c.m.) of astrocytes-microglia co-cultures stimulated or not with CX3CL1 (100 nM, 18 h). Representative blot is shown ($n = 12$). **(B–C)** CXCL16 up-regulation in microglia **(B)** and astrocytes **(C)** following CX3CL1 stimulation. Top: Western-blot analysis for mature and precursor (pre) CXCL16 species (Gough et al., 2004) in unstimulated and stimulated cells derived from co-cultures experiments. Representative blots are shown ($n = 6$). Bottom: qRT-PCR analysis for CXCL16 mRNA expression in microglia **(B)** and astrocytes **(C)** derived from co-cultures experiments treated or not with CX3CL1 for 4, 8, 18 h ($n = 8$). Data are expressed as mRNA fold increase normalized to GAPDH. Results represent the mean ± SEM. Statistical analysis: Student *t*-test * $p < 0.05$; ** $p < 0.001$ **(A, B–C** top panels); one-way ANOVA followed by Dunn's *post hoc* test **(B–C)** bottom panels * $p < 0.05$.

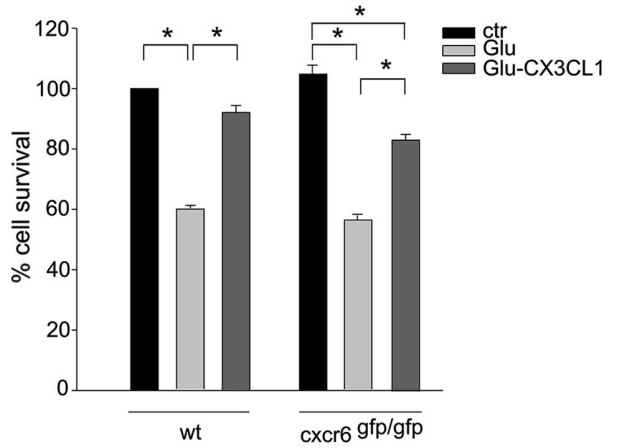

FIGURE 4 | CXCL16 contributes to CX3CL1 neuroprotection against Glu toxic insult. Glu-excitotoxic experiments were performed in *wt* and cxcr6 $^{gfp/gfp}$ mice hippocampal cultures treated or not with CX3CL1 (n = 8–10). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in *wt* control condition. Results represent the mean ± SEM. Statistical analysis: two-way ANOVA followed by Dunn'n *post-hoc* test * $p < 0.05$.

$n = 6$; $p < 0.05$). According to this result, the A3R inhibitor MRS1523 reduced CX3CL1 neuroprotection ($n = 5$; $p < 0.05$; **Figure 5B**). Moreover, as reported in **Figure 5C** in the presence of neutralizing αCCL2 Ab (but not with control IgG, both used at 3 μg/ml), CX3CL1 was not able to induce neuroprotection ($n = 4$; $p < 0.05$).

To verify the hypothesis that CX3CL1 could also induce the release of CCL2, that concur to neuroprotection, we stimulated microglia-astrocytes co-cultures or microglia with CX3CL1 for 18 h and measured CCL2 level in the c.m. **Figure 5D** shows a basal release of CCL2 that increases upon CX3CL1 stimulation in microglia-astrocyte co-culture ($n = 11$; $p < 0.001$ Rank sum Test) but not in microglia alone ($n = 7$; $p = 0.7$ Rank sum Test), suggesting that CX3CL1 acting on microglia, induces the release of CCL2 from astrocytes.

CX3CL1 NEUROPROTECTION AGAINST pMCAO IS REDUCED IN cxcr6$^{gfp/gfp}$ MICE

CX3CL1 is neuroprotective in pMCAO (Cipriani et al., 2011). To further confirm that CXCL16 contributes to CX3CL1 neuroprotection, *wt* and cxcr6$^{gfp/gfp}$ mice were i.c.v. injected with soluble CX3CL1, 30 min before induction of pMCAO: as reported in **Figure 6**, two-way ANOVA analysis reveals a significant difference between genotypes ($p < 0.001$), being the ischemic volume higher in cxcr6$^{gfp/gfp}$ mice vs. *wt* animals. CX3CL1 administration was effective in reducing ischemic volume in both genotypes ($p < 0.05$) but the reduction observed in cxcr6$^{gfp/gfp}$ mice was less pronounced being 10.9% ($n = 6$) vs. 25.6 % in *wt* ($n = 4$).

DISCUSSION

Glial cells, long thought to act as a mere "support" network, have been gaining increasing attention as crucial protagonists in a variety of neural functions including information processing but also cell viability. In the present paper we describe for the first time the ability of trasmembrane chemokines CX3CL1 and CXCL16 to drive molecular interplay between neurons, microglia and astrocytes in determining the neuroprotection against pMCAO and excitotoxic damage, showing that a concerted action of these cells is important to determine neuronal survival upon exposure to high level of Glu, a condition that normally occurs following ischemia (Castillo et al., 1996) but also in traumatic brain injuries (Zauner et al., 1996) or chronic neurodegenerative diseases (Shaw et al., 1995; Hallett and Standaert, 2004; Lipton, 2005).

In line with previous *in vitro* findings, we demonstrated that exogenous administration of soluble CXCL16 reduced brain ischemic volume following pMCAO; moreover we found that upon ischemic insult CXCL16 expression is increased in the ischemic hemisphere and that endogenous CXCL16 signaling is important *per se* to counteract brain damage, since in cxcr6$^{gfp/gfp}$ mice there is a significant increase in brain ischemic volume upon pMCAO. All together, these data indicate that CXCL16 represents a physiological mediator of self-protective mechanisms engaged by brain parenchyma to restrain cell damage following toxic insult. Upon brain ischemia, there is the simultaneous activation of destructive pathways leading to cell death but also of local protective mechanisms. Although the damaging effectors apparently prevail, evidences suggest that concomitant self-protective mechanisms might limit the resulting damage and set the stage for tissue repair and reorganization (Moskowitz et al., 2010; Iadecola and Anrather, 2011; **Figure 7**). Thus unveiling the molecular players that act in self-protective mechanism might provide new opportunity to treat brain pathologies.

Damaged neurons respond to neurotoxic insults releasing soluble factors that can be sensed by surrounding glia: CX3CL1, a chemokine selectively expressed by neurons in the nervous system, is one of such mediators being upregulated (Tarozzo et al., 2002; Zhu et al., 2009), cleaved and released upon ischemia and excitotoxic insult (Chapman et al., 2000; Limatola et al., 2005; Noda et al., 2011) and being able to drive neuroprotection (Limatola et al., 2005; Lauro et al., 2010; Cipriani et al., 2011). We reported here that, upon CX3CL1 stimulation, glial cells produce and release CXCL16, important for CX3CL1 neuroprotective effect. We speculate that CX3CL1 released from neurons upon ischemia might drive microglia-astrocytes cross-talk leading to CXCL16 increase. These data further corroborate the idea that, although the only direct targets of CX3CL1 are microglial cells, its neuroprotective effects are mediated by engagement of astrocytes that concur to limit excitotoxic cell death with the synergistic activity of adenosine (Catalano et al., 2013).

We do not know the mechanism that leads to the release of CXCL16 from glia, however it has been recently reported that activation of the purinergic receptor P2X7 induces CXCL16 shedding from RPMI8226 myeloma B cells (Pupovac et al., 2013). Hippocampal cells stimulation with CX3CL1 induces an increase in extracellular adenosine probably derived from released ATP, the effect being specifically blocked by the treatment with the ectonucleotidase inhibitor alpha-beta-methyleneadenosine 5′-diphosphate sodium salt (AOPCP) (Lauro et al., 2010). Since both astrocytes and microglia express P2X7 receptors, it could be hypothesized that CX3CL1 induces ATP release from microglia

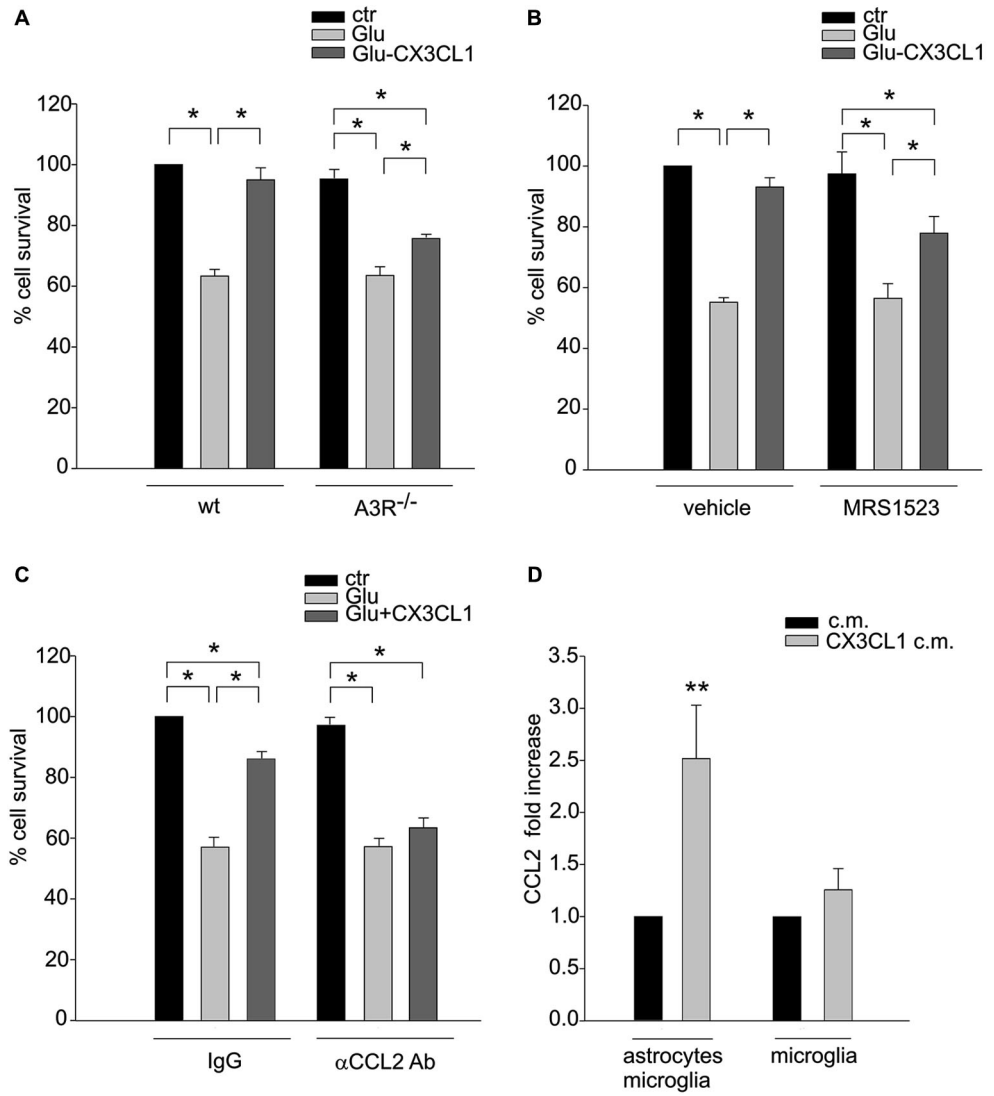

FIGURE 5 | A3R activity and astrocytic CCL2 concur to CX3CL1 neuroprotection. (A) Genetic deletion of A3R reduces CX3CL1 neuroprotection. Glu-excitotoxic experiments were performed in hippocampal cells derived from *wt* or $A3R^{-/-}$ mice ($n = 6$). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in *wt* control condition. **(B)** Pharmacological inhibition of A3R reduces CX3CL1 neuroprotection. Primary hippocampal cells were treated with A3R specific antagonists MRS1523 and used for Glu-excitotoxic experiments ($n = 5$). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in vehicle control condition. **(C)** Neutralization of CCL2 activity prevents CX3CL1 neuroprotection. Glu-excitotoxic experiment were performed in hippocampal cultures in the presence of neutralizing αCCL2 Ab (3 μg/ml) or control IgG (3μg/ml) ($n = 4$–6). Data are expressed as percentage of viable cells in treated cultures taking as 100% the number of viable cells in IgG control condition. **(D)** CX3CL1 triggers CCL2 release from astrocytes. Microglia-astrocytes co-cultures or microglia alone were treated with CX3CL1or vehicle, and the c.m. were collected after 18 h. CCL2 levels in the media were measured by ELISA ($n = 7$–11). Results represent the mean ± SEM. Statistical analysis: two-way ANOVA followed by Holm-Sidak *post-hoc* test * $p < 0.05$ **(A)**; one-way ANOVA followed by Holm-Sidak *post-hoc* test * $p < 0.05$ **(B–C)**; Student's *t*-test ** $p < 0.001$ **(D)**.

that, acting on P2X7 receptors, induces CXCL16 shedding from surrounding glial cells. A role for P2X7 in the release of neuroprotective mediators is in agreement with previous data showing that P2X7 activation reduces excitotoxic neuronal death, through TNF-α shedding from microglia (Suzuki et al., 2004).

Adenosine modulates neuron-glia communication (Boison et al., 2010) and can mediate neuroprotective effects through the activity of its own receptors: in this regards the activity of A1R is crucial to allow neuroprotection driven by CX3CL1, IL-6, oncostatin M (OSM), BDNF and erythropoietin (EPO) (Biber et al., 2008; Lauro et al., 2010; Moidunny et al., 2010). Also A3R activity can mediate neuroprotection since it has been shown that hypoxic conditions determine a wider neurodegeneration in $A3R^{-/-}$ mice (Fedorova et al., 2003) and i.c.v. injection of A3R selective agonist in mice reduces brain ischemic volume (Chen et al., 2006). In the present work, we confirmed an increased

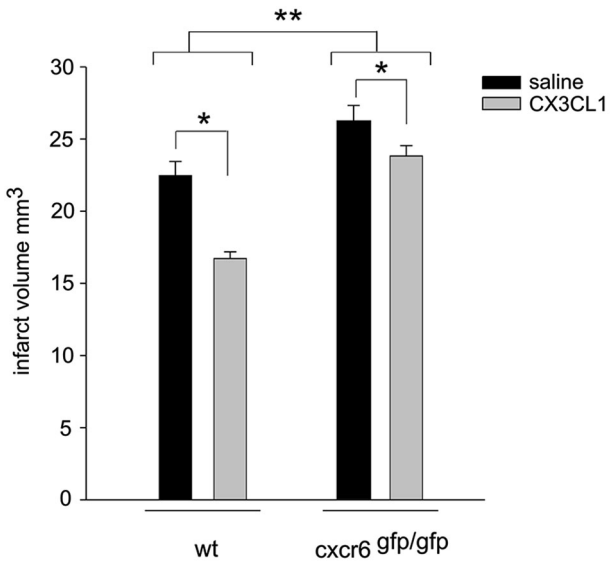

FIGURE 6 | CX3CL1 neuroprotection against pMCAO is reduced in cxcr6 $^{gfp/gfp}$ mice. Mice were i.c.v. injected with saline or CX3CL1 (70 pmol) 30 min before pMCAO and analyzed for ischemic volume 24 h later (n = 4–10). Results represent the mean ± SEM. Statistical analysis: two-way ANOVA followed by Holm-Sidak *post-hoc* test * $p < 0.05$; ** $p < 0.001$.

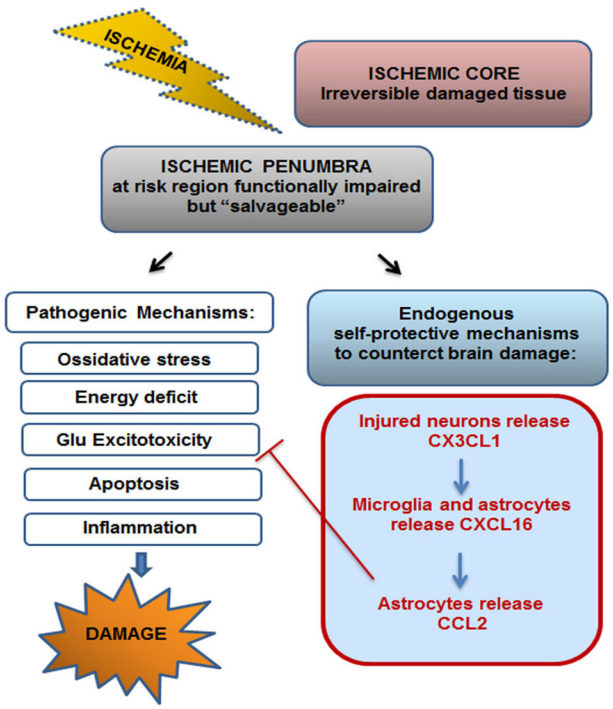

FIGURE 7 | Endogenous self-protective mechanism drived by CX3CL1–CXCL16-CCL2 upon ischemia. Ischemic insult drives irreversible brain damage in ischemic core, while in the surrounding region (ischemic penumbra) brain tissue results to be functionally compromised but potentially "salvageable". Upon ischemic insult both pathogenic and self-protective mechanisms occur in the penumbra. CX3CL1-CXCL16-CCL2 cascade, triggered by neurons-microglia-astrocytes cross-talk, represents one endogenous self-protective mechanism that contributes to the restriction of brain damage by counteracting Glu induced excitotoxicity.

ischemic volume in A3R$^{-/-}$ mice compared to *wt* mice and found that the ability of CXCL16 to reduce ischemic volume is less pronounced in these mice. This is in line with our previous *in vitro* findings, where we have shown that soluble CXCL16 is able to promote neuronal survival against excitotoxic damage depending on A3R activity (Rosito et al., 2012), and in particular the synergistic activity of CXCL16 and A3R on astrocytes causes the release of CCL2 that act as a key mediator of neuroprotection.

We speculate that upon ischemic insult, CXCL16 released from glia concurs to endogenous neuroprotective mechanism elicited by neuronal CX3CL1 since we found that CX3CL1-induced neuroprotection was reduced in cxcr6$^{gfp/gfp}$ mice; both genetic and pharmacological inactivation of A3R reduces CX3CL1 neuroprotection against Glu excitotoxic insult; CX3CL1 is able to increase the release of CCL2 from astrocytes; CCL2 activity is important for CX3CL1 protective effect.

Nevertheless, our data showed that impairment of CXCL16 or A3R signaling in transgenic animals reduced, but did not totally prevented CX3CL1 neuroprotection, indicating that the mechanism we here proposed represent only a portion of the neuroprotective mechanisms driven by CX3CL1.

The involvement of A1R in CX3CL1 neuroprotection (Lauro et al., 2010) strongly suggests that there must be at least another mechanism, independent from CXCL16, important to protect cells from Glu excitoxicity: accordingly we have recently published that the activity of Glu transporter GLT1 on astrocytes is increased by CX3CL1, with mechanisms requiring A1R activation and this event is also crucial for CX3CL1 neuroprotection (Catalano et al., 2013).

In conclusion, the present work highlights the role played by chemokines as key endogenous modulators of the cross-talk between cells of brain parenchyma, that drive physiological neuroprotective mechanisms. In particular we demonstrated the existence of chemokine induced chemokine release (CX3CL1-CXCL16-CCL2) mechanism that involves neurons, microglia and astrocytes and that represents an endogenous self-protective mechanism that upon brain ischemia can limit cell damage in the ischemic penumbra, by counteracting neuronal cell death due to Glu excitotoxicity (**Figure 7**).

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ELR(+) chemokine signaling in host defense and disease in a viral model of central nervous system disease

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Intracranial infection of the neurotropic JHM strain of mouse hepatitis virus (JHMV) into the central nervous system (CNS) of susceptible strains of mice results in an acute encephalomyelitis, accompanied by viral replication in glial cells and robust infiltration of virus-specific T cells that contribute to host defense through cytokine secretion and cytolytic activity. Mice surviving the acute stage of disease develop an immune-mediated demyelinating disease, characterized by viral persistence in white matter tracts and a chronic neuroinflammatory response dominated by T cells and macrophages. Chemokines and their corresponding chemokine receptors are dynamically expressed throughout viral infection of the CNS, influencing neuroinflammation by regulating immune cell infltration and glial biology. This review is focused upon the pleiotropic chemokine receptor CXCR2 and its effects upon neutrophils and oligodendrocytes during JHMV infection and a number of other models of CNS inflammation.

Keywords: chemokines, chemokine receptors, virus, neuroinflammation, demyelination

INTRODUCTION

Intracranial infection of susceptible mice with the JHM strain of mouse hepatitis virus (JHMV) causes an acute encephalomyelitis followed by a chronic demyelinating disease. JHMV, after initially infecting ependymal cells lining the ventricles, rapidly disseminates to astrocytes, oligodendroglia, and microglia throughout the brain and spinal cord (Wang et al., 1992). Although inflammatory virus-specific T cells are efficient in controlling viral replication through the secretion of IFN-γ and cytolytic activity, sterile immunity is not achieved. Viral protein and/or RNA persist within oligodendroglia and drive continual T cell and macrophage infiltration, leading to chronic neuroinflammation and demyelination. Histological features associated with viral persistence include the development of an immune-mediated demyelinating disease similar to the human demyelinating disease MS; both T cells and macrophages are critical mediators of disease severity, contributing to myelin damage (Cheever et al., 1949; Perlman et al., 1999).

Through the course of acute and chronic JHMV-induced neurologic infection, there is a coordinated expression of chemokines and chemokine receptors that regulate inflammation, contributing to both host defense and disease exacerbation. Among the chemokines expressed during infection are members of the ELR(+) chemokine family CXCL1 and CXCL2. CXCL1 and CXCL2 are potent chemoattractants for peripheral mononuclear cells (PMNs), binding and signaling through their receptor CXCR2 (Wolpe et al., 1989; Moser et al., 1990; Schumacher et al., 1992; Marro et al., 2012; Weinger et al., 2013). Moreover, PMNs have been shown to enhance central nervous system (CNS) inflammation by disrupting blood brain barrier (BBB) integrity in animal models of spinal cord injury (SCI; Tonai et al., 2001; Gorio et al., 2007), autoimmune demyelination (Carlson et al., 2008), and JHMV-induced encephalomyelitis (Zhou et al., 2003), while blocking or silencing of CXCR2 signaling mutes inflammation and tissue damage in mouse models in which PMN infiltration is critical to disease initiation (Kielian et al., 2001; Belperio et al., 2005; Londhe et al., 2005a,b; Strieter et al., 2005; Gorio et al., 2007; Wareing et al., 2007; Carlson et al., 2008).

CXCR2 is also expressed by oligodendrocytes (Omari et al., 2005), and CXCL1 promotes the proliferation and positional migration of oligodendrocyte precursor cells (Robinson et al., 1998; Robinson and Franic, 2001; Tsai et al., 2002; Filipovic and Zecevic, 2008). Further, both CXCR2 and CXCL1 are expressed within active MS lesions (Omari et al., 2005, 2006). How and whether CXCR2 and its cognate ligands regulate immune and glial cell function during acute and chronic disease of the CNS is the focus of this review.

ELR(+) CHEMOKINE SIGNALING PROMOTES PMN INFILTRATION INTO THE CNS DURING ACUTE JHMV INFECTION

Following JHMV infection, mRNA for the chemokine receptor CXCR2 and its associated ligands CXCL1 and CXCL2 are significantly upregulated within the acutely infected CNS, peaking at 3 days pi (**Figure 1A**). CXCL1 expression was localized to astrocytes (GFAP-positive) within the parenchyma and associated with the microvasculature (**Figure 1B**), consistent with previous observations (Lane et al., 1998; Omari et al., 2006; Rubio and Sanz-Rodriguez, 2007). The expression of the CXCR2 ligands within the CNS closely paralleled neutrophil emergency release into the circulation and infiltration into the CNS; CXCR2-expressing neutrophils were detectable as early as 1 day pi and peaked at 3 days pi within both the periphery and the CNS (Hosking et al., 2009).

To determine whether CXCR2—signaling controlled neutrophil infiltration into the CNS, JHMV-infected mice were treated with either CXCR2 antiserum or control serum (NRS). Neutralization of CXCR2 almost completely abrogated neutrophil infiltration into the CNS (**Figures 1C,D**). Without infiltrating neutrophils, permeabilization of the blood-brain barrier was impaired (Hosking et al., 2009) and subsequent inflammatory cell infiltration was significantly reduced. Mice treated with CXCR2 neutralizing antiserum were incapable of controlling viral replication, and 100% of all infected mice succumbed to viral infection within 11 days and this was associated with an impaired ability to control CNS viral replication (**Figures 1E,F**). Moreover, total and virus specific $CD4^+$ and $CD8^+$ T cell infiltration into the CNS was diminished. Notably, CXCR2 neutralization did not alter the peripheral generation of virus-specific T cells, indicating that the increased mortality and diminished ability to control viral infection within the CNS is likely associated with the dampened access of T cells into the CNS parenchyma (Hosking et al., 2009). Collectively, these data demonstrate that during viral infection of the CNS, CXCR2 and its associated chemokines function to non-redundantly attract neutrophils into the CNS, where they are required to permeabilize the blood-brain barrier, thus facilitating subsequent inflammatory cell infiltration and control of viral replication.

ELR(+) CHEMOKINE SIGNALING AND NEUTROPHILS IN OTHER MODELS OF CNS INFLAMMATION

Neutrophils are amongst the earliest inflammatory infiltrate into the CNS following experimental autoimmune encephalitis (EAE) induction, and their presence precedes axonal damage, demyelination, and clinical disease (Carlson et al., 2008; Soulika et al., 2009; Wu et al., 2010). Neutralization of either CXCR2 (Carlson et al., 2008) or CXCL1 (Roy et al., 2012) potently reduces neutrophil infiltration into the CNS and reduces BBB permeability, thereby significantly delaying the onset and peak of clinical symptoms. Neutrophils also infiltrate into the CNS during the first week following cuprizone feeding, and their early presence in the CNS is absolutely necessary for the subsequent demyelination observed within the corpus callosum (Liu et al., 2010a). CXCR2 deficient mice or bone marrow chimeric mice, where myeloid cells lack CXCR2, or neutrophil-depleted mice are resistant to cuprizone induced demyelination (Liu et al., 2010a). Interestingly, although neutrophils are also critical for lymphocytic choriomeningitis virus (LCMV)- and pilocarpine-induced BBB permeabilization and subsequent seizures (Fabene et al., 2008; Kim et al., 2009), they are dispensable for seizures during Theiler's murine encephalomyelitis virus (TMEV; Libbey et al., 2011), underlining the fact that neutrophils are not the only cell type capable of mediating permeabilizing the BBB. To this point, resident monocytes, astrocytes, and $CD8^+$ T cells are all capable of direct permeabilization (Savarin et al., 2010, 2011; Johnson et al., 2012). Nevertheless, CXCR2-directed neutrophil infiltration into the CNS is a key determinate for subsequent inflammatory cell infiltration in a variety of CNS models of viral infection, demyelination, and autoimmunity.

ELR(+) CHEMOKINE SIGNALING PROMOTES OLIGODENDROGLIA SURVIVAL DURING CHRONIC JHMV-INDUCED DEMYELINATION

How chemokine receptor signaling contributes to chronic neurologic diseases has largely been considered within the context of targeted leukocyte recruitment into the CNS (Liu et al., 2000, 2001a,b; Glass and Lane, 2003; Hosking et al., 2009). However, numerous resident cell types of the CNS also express chemokine receptors under non-inflammatory and inflammatory conditions (reviewed in Bajetto et al., 2001; Ubogu et al., 2006), indicating that these cells are capable of responding to specific chemokine ligands. Thus, chemokine signaling may participate in either repair and/or exacerbation of pathology following insult, injury, or infection of the CNS (Liu et al., 2001b; Kerstetter et al., 2009; Omari et al., 2009).

Following JHMV infection, mRNA transcripts for CXCR2 as well as its ligands CXCL1 and CXCL2 are significantly upregulated, persisting until at least 21 days pi within the spinal cord (**Figure 2A**). CXCL1 expression was localized to GFAP+ astrocytes within the white matter (**Figure 2B**), suggesting that CXCR2, besides attracting neutrophils during early acute viral infection, may also alternatively function during chronic demyelination. To determine whether CXCR2 signaling was beneficial or pathogenic, mice persistently infected with JHMV were treated with anti-CXCR2 or control serum (NRS) from day 12–20 p.i. CXCR2 neutralization significantly delayed spontaneous clinical recovery (**Figure 2C**). Correspondingly, spinal cords from anti-CXCR2 treated mice revealed significantly greater areas of demyelination (**Figures 2D,E**). Importantly, CXCR2 neutralization during chronic JHMV infection did not affect inflammatory cell infiltration into the CNS (Hosking et al., 2010).

CXCR2 neutralization was also associated with an increase of apoptotic oligodendrocytes and oligodendrocyte precursor cells within white matter tracts of the spinal cord (**Figure 2F**; Hosking et al., 2010). To determine whether or not CXCR2

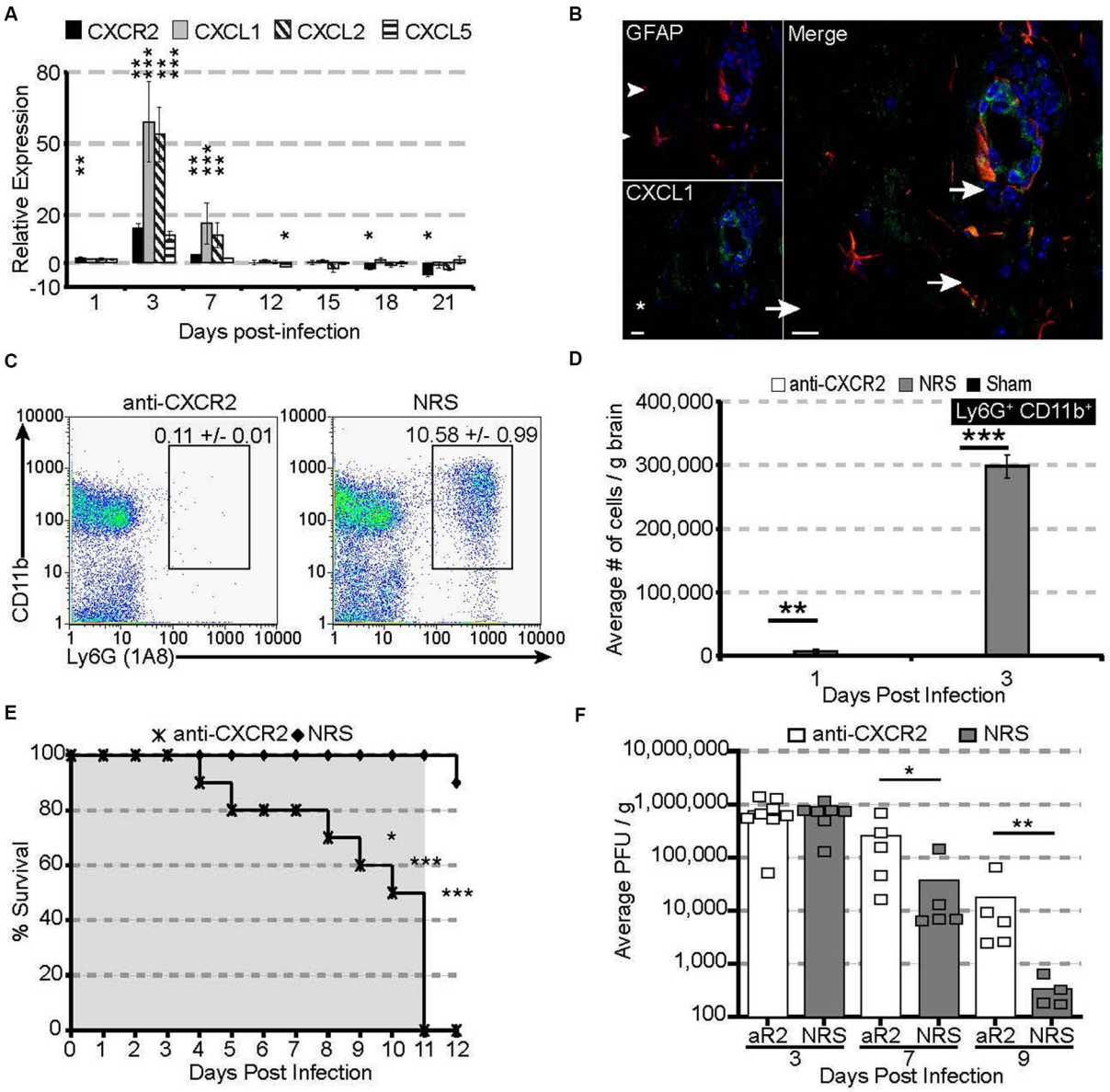

FIGURE 1 | CXCR2 drives neutrophil infiltration into the CNS during acute JHMV infection. C57BL/6 mice were infected with JHMV and their brains removed at the indicated time points. **(A)** mRNA for CXCR2, CXCL1, and CXCL2 are upregulated within the brains of JHMV infected mice. **(B)** Immunofluorescence staining reveals that the majority of CXCL1 (green) co-localizes with GFAP+ (red) astrocytes. **(C)** Representative FACS plots depicting the average frequency of neutrophils at day 3 are shown in panel. **(D)** Neutralization of CXCR2 blocks neutrophil ($Ly6G^+CD11b^+$) infiltration into the CNS. **(E)** CXCR2 neutralization results in 100% morality by day 11 pi (shaded area indicates the treatment period) and **(F)** elevated viral loads within the brains of treated mice. *NRS* = normal rabbit serum treated mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to NRS-treated mice.

could directly prevent JHMV-mediated apoptosis, cultured oligodendroglia were infected with JHMV *in vitro* and treated with varying concentrations of CXCL1. In accordance with previous observations (Liu et al., 2003, 2006; Liu and Zhang, 2005, 2007), JHMV—infected oligodendrocytes readily underwent apoptosis (**Figure 2G**), and western blotting confirmed activated caspase 3, cleaved poly ADP ribose polymerase (PARP) (a caspase 3 target), and muted expression of Bcl-2 (**Figure 2I**). CXCL1, in a dose-dependent manner, prevented JHMV-mediated apoptosis (**Figure 2G**). Moreover, activated caspase 3 and cleaved PARP were undetectable in CXCL1-treated cultures (**Figure 2I**). Notably, CXCL1 was incapable of rescuing CXCR2 deficient cultures from JHMV-mediated apoptosis (**Figures 2H,I**). CXCR2 also prevents IFNγ-and CXCL10- mediated apoptosis of murine or human oligodendroglia cultures (Tirotta et al., 2011, 2012). Collectively, these data suggest that CXCR2, during chronic viral infection of the CNS, prevents oligodendrocyte

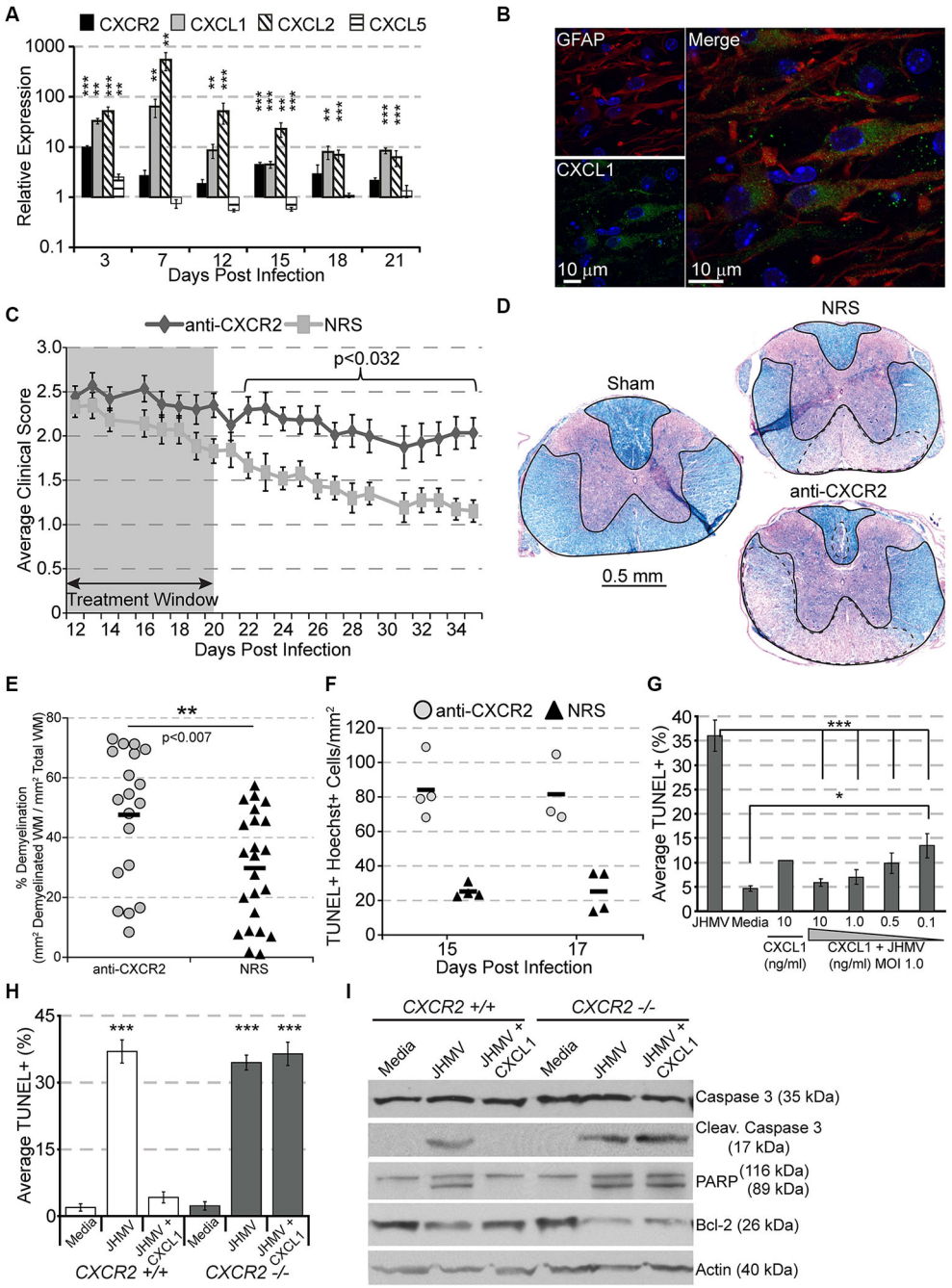

FIGURE 2 | CXCR2 promotes spontaneous recovery and oligodendrocyte survival during chronic JHMV infection. C57BL/6 mice were infected with JHMV and their spinal cords removed at the indicated time points. **(A)** mRNA for CXCR2, CXCL1, and CXCL2 are upregulated within the spinal cords of JHMV infected mice. **(B)** Immunofluorescence staining reveals that the majority of CXCL1 (green) co-localizes with GFAP-positive (red) astrocytes within the spinal cord white matter. **(C)** Neutralization of CXCR2 (from day 12–20 pi) delays clinical recovery from chronic JHMV infection. **(D and E)** Mice receiving CXCR2 antiserum had significantly greater total areas of demyelination within the spinal cord. Representative luxol fast blue stained spinal cords are shown in panel **(D)** with the total (solid line) and demyelinated (dashed line) white matter indicated. **(F)** Significantly ($p < 0.001$) increased numbers of apoptotic (TUNEL+) cells were observed within the spinal cords of anti-CXCR2 treated mice. **(G)** CXCL1, in a dose-dependent manner, protects oligodendrocytes from apoptosis, and **(H)** CXCR2-deficienct oligodendrocyte-enriched cultures are not protected from apoptosis. **(I)** Protein lysates from CXCR2-sufficient and CXCR2-deficient oligodendrocyte cultures were assessed via western blot for total caspase 3, activated caspase 3, PARP, Bcl-2, and actin expression. *NRS* = normal rabbit serum treated mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to NRS-treated mice.

apoptosis and promotes clinical recovery from viral induced demyelination.

ELR(+) CHEMOKINE SIGNALING AND OTHER MODELS OF CNS INFLAMMATION AND DEMYELINATION

The role for CXCR2 signaling during EAE and a variety of toxin—induced demyelination models has also been studied. Raine and colleagues (Omari et al., 2009) have shown that CXCL1, when inducibly expressed by astrocytes after the onset of EAE, reduces peak disease severity, reduces total demyelination, and increases the onset of remyelination. Moreover, transgenic CXCL1 was associated with greater proliferation (presumably of oligodendrocyte precursors) throughout the spinal cord white matter (Omari et al., 2009). Conversely, Ransohoff and colleagues (Liu et al., 2010b) have demonstrated, using a series of bone marrow chimeras, that parenchymal CXCR2 deficiency on radio-resistant cells promotes faster recovery from EAE, cuprizone—induced demyelination, and *in vitro* lysotecithin-induced demyelination. Notably, initial clinical severity, inflammation, and/or demyelination in all three models of demyelination and repair were similar regardless of whether parenchymal cells possessed CXCR2; accelerated recovery was associated with initial increases in oligodendrocyte precursor cells, followed by an increased density of mature myelinating oligodendrocytes (Liu et al., 2010b). Similar results were observed following CXCR2 chemical anatagonism during EAE and *in vivo* lysolecithin-induced demyelination (Kerstetter et al., 2009).

PERSPECTIVES

The JHMV-induced model of viral-induced encephalomyelitis provides an important tool in defining molecular and cellular mechanisms that regulate neuroinflammation during both host defense and disease progression. Our research on chemokines and chemokine receptors has revealed important roles for these molecules in orchestrating CNS inflammation in response to JHMV infection. We and others have found unique and pleiotropic roles for ELR+ chemokine signaling via CXCR2 in moderating neutrophil infiltration and protecting oligodendroglia from apoptosis in response to exposure to virus and proinflammatory cytokines. Ongoing research in our laboratory continues to focus on the role of ELR(+) chemokine signaling on oligodendroglia during JHMV-induced neuroinflammation. It will be important to analyze the effects of selectively ablating CXCR2 on oligodendroglia during JHMV-induced demyelination, while simultaneously manipulating the cellular sources of ELR-positive chemokines in the CNS that may promote neuroprotection during chronic JHMV-induced disease.

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Chemokine receptors as important regulators of pathogenesis during arboviral encephalitis

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The central nervous system (CNS) is a highly complex network comprising long-lived neurons and glial cells. Accordingly, numerous mechanisms have evolved to tightly regulate the initiation of inflammatory responses within the brain. Under neuroinflammatory conditions, as in the case of viral encephalitides, the infiltration of leukocytes is often required for efficient viral clearance and recovery. The orchestration of leukocyte migration into the inflamed CNS is largely coordinated by a large family of chemotactic cytokines and their receptors. In this review, we will summarize our current understanding of how chemokines promote protection or pathogenesis during arbovirus induced encephalitis, focusing on neurotropic flaviviruses and alphaviruses. Furthermore, we will highlight the latest developments in chemokine and chemokine receptor based drugs that could have potential as therapeutics and have been shown to play a pivotal role in shaping the outcome of disease.

Keywords: chemokine receptors, flaviviruses, alphaviruses, leukocyte infiltration, antagonists

INTRODUCTION

Arboviruses (**ar**thropod-**bo**rne viruses) are a significant cause of human morbidity and mortality and have worldwide distribution. In recent years, these viruses have become an increasing public health concern due to climate change, increased globalization, and other environmental factors, that have caused their unexpected geographic expansion and increased the frequency of outbreaks (Gubler, 1996). The World Health Organization has estimated that arboviral infections constitute ∼30% of all emerging infectious diseases in the past decade (Jones et al., 2008). The most recent and well-documented examples include the introduction and spread of WNV in North America in 1999 and the continuing emergence of CHIKV in the regions of the Indian Ocean in 2005/2006 (Hayes et al., 2005; Bonn, 2006).

Neurotropic arboviruses have the capacity to enter the CNS and cause inflammation and severe neurologic sequelae in humans. Many of these viruses are members of the *Flavivirus* (*Flaviviridae* family) and *Alphavirus* (*Togaviridae* family) genera. The main perpetrators of arboviral infections in humans include JEV, with 30,000–50,000 cases reported annually, WNV, and TBEV (Campbell et al., 2011). Mosquito-borne alphaviruses are also important causes of encephalomyelitis and include WEEV, EEEV, and VEEV. SFV and SINV are neurotropic viruses that do not usually cause encephalitis in humans, but are studied frequently in mice as model systems for alphavirus-induced encephalomyelitis.

Acute viral encephalitis is a life-threatening condition that is characterized by the presence of leukocytes within the brain parenchyma. Viral replication within the CNS can lead to neuronal damage and results in apoptosis and necrosis of these cells. As part of innate and adaptive immune responses to viral replication, a large number of leukocytes infiltrate the CNS, and the cell types and composition of the inflammatory response can vary greatly between individuals and between pathogens. The large influx of leukocytes into the normally immune-sheltered CNS is required for recovery and clearance of virus but is often associated with neuropathology (Hosking and Lane, 2010; Ransohoff and Engelhardt, 2012).

Chemokines play a pivotal role in the attraction of leukocytes into the CNS, and it is imperative to understand their cell-type specific role in pathogenesis in order to develop novel immunotherapeutics and predict the impact of chemokine receptor antagonism in humans. Chemokines and their receptors comprise a large superfamily of proteins that can be categorized into four subfamilies based on the position of the first two cysteines within the first amino terminal cysteine motif: CC, CXC, XC, and CX_3C (Zlotnik and Yoshie, 2000). All chemokine receptors are G-protein coupled receptors, containing a seven-transmembrane domain that interacts with the appropriate chemokine upon binding. Chemokines and chemokine receptors have been shown to have pivotal roles in organizing and coordinating complex immune system functions (Zlotnik and Yoshie, 2012). Many studies have been conducted in the past to elucidate the role of chemokines during viral encephalitis. In this review, we will summarize the role of chemokines and their receptors specifically during arbovirus induced encephalitis. In particular, we will focus on WNV, JEV, TBEV, SFV, and SINV, as

Abbreviations: BBB, blood–brain barrier; BCSFB, blood–cerebrospinal fluid barrier; CHIKV, Chikungunya virus; CNS, central nervous system; CSF, cerebrospinal fluid; DC, dendritic cell; EEEV, Eastern equine encephalitis virus; FDA, Food and drug administration; HIV, human immunodeficiency virus; iNOS, inducible nitric oxide synthetase; JEV, Japanese encephalitis virus; MHC, major histocompatibility complex; MVEV, Murray valley encephalitis virus; SCID, severe combined immunodeficiency; SFV, Semliki Forest virus; SINV, Sindbis virus; TBEV, tick borne encephalitis virus; TLR, toll-like receptor; TNF-α, tumor necrosis factor alpha; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus; WNV, West Nile virus.

these pathogens are the most studied in the context of chemokine-mediated leukocyte infiltration into the virally infected CNS in both mouse models and humans. Furthermore, we will also highlight chemokine receptor based drugs that are either approved or in development for human use, as well as chemokine specific antibodies, and their anticipated effect in the context of human arboviral encephalitis.

IMMUNE RESPONSES IN THE CNS DURING ARBOVIRAL ENCEPHALITIS

From an immunological point of view, the CNS is a unique compartment due to the following features: lack of antigen presenting cells, low expression of MHC I and MHC-II, lack of lymphatic vessels within the brain, absence of resident DC, BBB, and BCSFB that restrict entry of cells and substances into the CNS (Ransohoff et al., 2003). If the BBB is compromised due to infection or inflammation, immune cells are able to infiltrate the brain (Rivest, 2009). Despite the mostly effective host responses during early stages of viral infection, controlling viral spread within the CNS requires the influx of peripheral leukocytes that can often cause profound damage to neurons and glial cells. Therefore, immune responses within the host must be balanced as to prevent damage to delicate and mostly non-renewable neurons.

Neurotropic arboviruses replicate in the periphery prior to entry and replication in the tissue of the CNS. Within peripheral organs or lymphoid tissues, the elicited immune response is often sufficient to prevent viral entry into the CNS. In fact, most infections with flaviviruses are asymptomatic/subclinical, with no evidence of neuroinvasion (Mostashari et al., 2001). However, if the virus enters the CNS, the infected target cells as well as bystander cells produce numerous chemokines and cytokines, which in turn initiate neuroinflammation (Neumann, 2001). Based on several RNA based assays, some of the chemokines produced within the CNS during arboviral encephalitis are CCL1–5, CCL7, CCL8, CCL12, CXCL1, CXCL2, and CXCL9–13 (Gupta and Rao, 2011; Yang et al., 2011; Metcalf et al., 2013; Palus et al., 2013; Michlmayr et al., 2014). In particular CCL2–CCL5 and CXCL10 are consistently and highly induced during JEV, WNV, TBEV, SFV, and SINV infection. In addition to infected neurons, activated astrocytes and microglia are also a major source of chemokines within the inflamed brain. Our study and a study by Shirato et al. has revealed that the extent of chemokine expression during WNV infection is dependent on viral strain and severity of the disease (Shirato et al., 2004; Michlmayr et al., 2014). Another study with TBEV has shown that mice highly susceptible to TBEV infection display a higher fold induction of chemokine transcripts and low levels of neutralizing antibodies compared to mice with low susceptibility to TBEV infection. Thus, the extent of the host response may be positively correlated with pathogenesis of TBEV infection in mice (Palus et al., 2013).

The leukocytic infiltrate and its role within the CNS during viral encephalitis is dependent on numerous factors, including the infected cell type, the route of infection, and the strain and inoculum of virus. Evaluation of human and mouse CNS tissue indicates that neutrophils, $CD4^+$ and $CD8^+$ T-cells, and monocytes/macrophages are typically present during viral infection (Parsons and Webb, 1982; Johnson et al., 1986; Williamson et al., 1991; Holub et al., 2002; Samuel and Diamond, 2006). Currently, the role of neutrophils during neurotropic viral infections, both in the periphery and within the CNS, is unclear. Antibody depletion of neutrophils results in variable outcomes, depending on the virus used and timing of administration; the receptors used to enter the CNS have not been studied. T-cells, in particular $CD8^+$ T-cells, are a critical component of the cellular infiltrate into the brain (Shrestha et al., 2005; Sitati and Diamond, 2006), and blockade of T-cell entry into virally infected brains often results in changes in CNS viral loads and survival in mice. Whether T-cells are protective or pathogenic during viral encephalitis depends on the virus. Whereas T-cells play a predominantly protective role during WNV infection by mediating viral clearance in a perforin and granzyme-dependent manner (Shrestha et al., 2005; Sitati and Diamond, 2006), T-cells play a pathogenic role during TBEV infection, since $CD8^{-/-}$ mice and SCID mice showed increased survival (Růžek et al., 2009). For monocytes/macrophages, it is clear that these cells do function in regulating pathogenesis, but this appears to be highly dependent on the strain of virus used, as well as the model of infection. Thus, it appears that the biological roles of specific cell subsets and the complex signals involved in their migration and function are highly dependent on the context of infection, as well as the virus itself. In this review, we will summarize the current understanding of the chemokine receptors CCR2, CCR5, CXCR2, CXCR3, and CXCR4 during flavivirus and alphavirus infection, focusing on the effect of therapeutic blockade of these receptors using small molecule receptor antagonists or chemokine neutralizing antibodies.

THE ROLE OF CHEMOKINE RECEPTORS IN THE PATHOGENESIS OF ARBOVIRAL ENCEPHALITIS

CCR2

CCR2 is often considered to be a receptor associated with monocyte trafficking, which is consistent with its high and uniform expression on $Ly6c^{hi}$ "inflammatory" monocytes (Mack et al., 2001; Geissmann et al., 2003). This receptor is also found on subsets of activated T-cells, DC, and NK cells. In humans, a functionally equivalent "inflammatory" monocyte subset, identified as $CD14^+CD16^-$ also uniformly expresses CCR2 (Auffray et al., 2009). The primary and specific ligand for CCR2 is CCL2, but this receptor can also bind to ligands CCL7 and CCL12/CCL13 (Zlotnik and Yoshie, 2000). CCR2 has been postulated to be critical for the migration of monocytes into tissues during various inflammatory conditions (**Figure 1**). Recently, a novel role for CCR2 in regulating monocyte egress from the bone marrow under homeostatic and inflammatory conditions has been identified (Serbina and Pamer, 2006; Tsou et al., 2007). Thus genetic deficiency of CCR2 results in severe monocytopenia that could account for the partial or entire loss of these cells in inflamed tissues. The mechanism by which CCR2 modulates monocyte egress from the bone marrow into blood involves stromal cell sensing of TLR ligands, including those involved in viral sensing within the bone marrow. This results in the production of CCL2 within hours after infection, thus providing a mechanism by which systemic infections can trigger monocyte release (Shi et al., 2011). Thus, CCR2 and its ligands CCL2 and CCL7 are critical for modulating circulating monocyte numbers, and the additional role of this receptor in

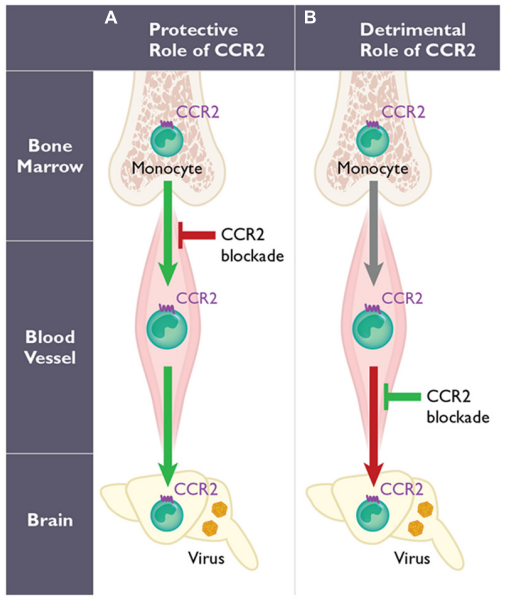

FIGURE 1 | The dual role of CCR2-expressing monocytes during West Nile virus (WNV) infection. The function of CCR2 on monocytes may involve two distinct steps: egress from the bone marrow induced early following WNV infection, and their migration into the WNV-infected central nervous system (CNS). However, the role of CCR2-expressing monocytes is highly dependent on the mode of infection utilized in mouse models. With peripheral infection **(A)**, WNV induces a monocytosis within several days post infection. Genetic deficiency or pharmacological blockade of CCR2 in mice (red arrow) results in monocytopenia and leads to decreased monocyte infiltration into the CNS, enhanced viral titers in the brain and increased mortality. Therefore, CCR2-mediated monocytosis and the subsequent migration of monocytes into the inflamed CNS are protective (green arrows). In contrast using an intranasal infection model of WNV infection **(B)**, the migration of CCR2-expressing monocytes into the CNS has also been shown to promote pathogenesis (red arrow) in mice. Thus, blocking CCR2 using anti-CCL2 antibodies in this model prolongs survival (green) in mice.

mediating migration from the blood into inflamed organs like the CNS, may be context-dependent (Shi et al., 2011).

Monocytes appear to have a critical role during WNV infection, although whether these cells function to protect or promote pathogenesis is unclear. Several studies have evaluated the role of monocytes using clodronate-loaded liposomes. In the first study, mice were infected intraperitoneally using a non-neuroinvasive strain of WNV, and monocyte depletion resulted in increased viremia, enhanced viral entry into the CNS, and increased mortality (Ben-Nathan et al., 1996). Another study, using subcutaneous inoculation of a neurotropic strain of WNV, showed a significant increase in peripheral and CNS viral loads and increased mortality following clodronate treatment (Purtha et al., 2008). Both studies imply that monocytes play a protective role in the context of WNV encephalitis (**Figure 1**). In a third study that utilized a lethal intranasal model and a non-neurotropic strain of WNV, depletion of peripheral monocytes, after treatment with clodronate, resulted in a reduction of $CD11b^+$ cells in the WNV-infected brain during WNV encephalitis (Getts et al., 2008). The authors blocked $Ly6c^{hi}$ monocyte migration into the CNS using an anti-CCL2 antibody and found a prolonged survival time compared to isotype-treated mice (**Table 1**), suggesting that inflammatory monocytes may function pathogenically during WNV encephalitis in this model (**Figure 1**).

Using *Ccr2*-deficient mice, which are monocytopenic, we observed higher mortality and enhanced viral titers in the CNS during WNV infection compared to wild type mice, supporting a protective role of CCR2 (Lim et al., 2011). Detailed analysis of monocytosis revealed that WNV induces an approximate fivefold increase in $Ly6c^{hi}$ monocytes in the blood within the first 5 days post infection. This response was found to be entirely dependent on CCR2, since $Ccr2^{-/-}$ mice showed no increase in monocyte levels in the blood throughout the course of infection. Within the CNS, a specific loss of $Ly6c^{hi}$ monocytes was observed, while other infiltrating leukocyte subsets ($CD4^+$ and $CD8^+$ T-cells, neutrophils, and NK cells) remained unchanged compared to wild type controls, correlating the specific loss of inflammatory monocyte accumulation in the CNS with increased mortality. Adoptive transfer of monocytes into WNV-infected $Ccr2^{-/-}$ mice showed that both CCR2-expressing and CCR2-deficient monocytes were capable of entering the CNS, suggesting monocyte migration into the CNS is CCR2-independent.

Because of the significant role of CCR2 in monocytosis during WNV infection, the induction of its ligands, CCL2, CCL7, and CCL12/CCL13 is implicit. Indeed, CCL2 is detected in the plasma of WNV-infected blood donors during the acute phase of infection, and numerous studies have also shown strong induction of CCL2 *in vitro* (Tobler et al., 2008; Semple et al., 2010; Hussmann et al., 2013). Among the CCR2 ligands, it appears that CCL2 and CCL7 play a dominant role in regulating CCR2-mediated monocytosis, while CCL12 was dispensable at the steady state (Tsou et al., 2007). It is unclear at the moment which of these chemokines (or both) is involved in WNV-induced monocytosis, and whether migration from the blood into the CNS requires either or both of these ligands.

Very little is known regarding the role of monocytes or CCR2 during other flavivirus or alphavirus infections. In the context of JEV and TBEV infection, CCL2 has been detected in the plasma and CSF of patients (Michałowska-Wender et al., 2006; Gupta et al., 2010); however, no studies have evaluated the role of monocytes or CCR2 in the context of these infections in mice. For alphavirus infections, we recently showed that the CCR2 ligand, CCL2, is highly inducible in the brain during SFV infection in mice (Michlmayr et al., 2014). The extent of CCL2 upregulation was correlated with the virulence of the strain, with the virulent strain of SFV inducing a >3000-fold induction above healthy control brains versus an ~90-fold induction using the avirulent strain of SFV. Interestingly, the composition of the CNS infiltrate differed greatly between these two strains, with the avirulent strain inducing $Ly6C^{hi}$ monocyte infiltration followed by a large influx of T-cells at the peak of infection. Conversely, the virulent strain of SFV induced an early and large influx of monocytes, with mice succumbing to infection by day 6, prior to when T-cells typically appear in the CNS. To investigate the role of CCR2 in this model, we infected *Ccr2*-deficient mice with virulent SFV but found no significant change in survival (data not shown). We also evaluated the therapeutic potential of CCR2 blockade

Table 1 | Inhibitors of chemokines/receptors and their role in arboviral encephalitis pathogenesis.

Blockade	Compound or antibody	Pathogen model	Role of antagonist	Reference
Receptor antagonists				
CCR2	RS504393	SFV	No effect	Michlmayr et al. (2014)
CCR5	DAPTA	SFV	No effect	Michlmayr et al. (2014)
CXCR3	Compound 21	SFV	No effect	Michlmayr et al. (2014)
CXCR3 & CCR2	Compound 21 and RS504393	SFV	Beneficial	Michlmayr et al. (2014)
CXCR4	AMD3100	WNV	Beneficial	McCandless et al. (2008)
Antibodies				
CXCL10	1F11 or 1B9	WNV	Pathogenic	Klein et al. (2005)
CCL2	2H5	WNV	Beneficial	Getts et al. (2008)

The role of chemokine/receptor blockade can be either beneficial, pathogenic or have no effect on disease outcome in murine models of encephalitis.

during SFV infection using a CCR2-specific antagonist, RS504393, which is a highly selective small molecule inhibitor (Mirzadegan et al., 2000; Furuichi et al., 2003). Treatment of SFV-infected mice twice per day, starting on day 3, resulted in no significant change in survival compared to untreated mice (Michlmayr et al., 2014). In both *Ccr2*$^{-/-}$ mice and RS504393-treated mice, drastically reduced monocyte numbers were observed in the CNS compared to control-infected mice. Interestingly, brain viral titers in *Ccr2*$^{-/-}$ and RS504393-treated versus control mice were not significantly altered (**Table 1**), suggesting that monocytes may not contribute to viral clearance in the CNS (Michlmayr et al., 2014). Together, these results suggest that monocytes, although they migrate into the CNS during SFV infection of mice, may not impact pathogenesis. More work is required to fully understand monocyte migration and function during SFV and other alphavirus infections.

CCR2 receptor antagonists

There is great interest in developing a CCR2-specific antagonist due to pathogenic roles of monocytes in a wide range of inflammatory diseases (Bachelerie et al., 2013). Currently, several small molecule receptor antagonists have made it successfully into clinical trials, including JNJ-17166864 developed by Johnson and Johnson, CCX140 from ChemoCentryx, and a CCR2-neutralizing antibody from Millenium (Hou et al., 2008; Hanefeld et al., 2012; Bachelerie et al., 2013). Based on our current understanding of how monocytes function during viral infection of the CNS, it would be expected that blocking monocyte entry into the CNS via CCR2 blockade, either by preventing their egress from the bone marrow or their migration into the CNS, could be detrimental to the patient during natural infection. However, our studies using SFV infection as a model of neurotropic alphavirus infection suggest that CCR2 blockade in this context may have no effect (Michlmayr et al., 2014).

CCR5

Due to its role as an HIV-1 co-receptor, CCR5 is one of the most highly studied chemokine receptors to date (Deng et al., 1996; Dragic et al., 1996). This receptor is mainly expressed on subsets of activated T-cells, NK-cells and myeloid cells (**Figure 2**), including monocytes, DCs, and microglia (Mack et al., 2001). Until recently, the function of CCR5 in host defense in humans was thought to be redundant with other closely related receptors, since individuals genetically deficient for CCR5 showed no increased susceptibility to infectious agents (Lim et al., 2006). However, recent studies have provided evidence that CCR5 may have a neuroprotective role during acute flaviviral infections of the CNS in both mice and humans.

The CCR5 ligands, CCL3, CCL4, and CCL5 have been shown to be among the most highly upregulated chemokines in the CNS during WNV, TBEV, JEV, and SFV infection in mice and humans (Glass et al., 2005; Klein et al., 2005; Palus et al., 2013; Michlmayr et al., 2014). The source of these ligands *in vivo* is unclear; however, *in vitro* studies, using primary cell cultures, suggest that, at least for CCL5, the major source could be astrocytes and possibly microglia (Cheeran et al., 2005; Chen et al., 2011; Hussmann and Fredericksen, 2014). The extent of CCL5 production may be dependent on the pathogenic potential of the virus, with astrocytes producing significantly higher levels of CCL5 when encountering a more virulent strain of WNV (Hussmann and Fredericksen, 2014). In WNV-infected plasma samples from blood donors during the viremic phase of infection, none of the CCR5 ligands were elevated above control levels (Tobler et al., 2008). However, in the context of JEV infection, CCL5 levels detected in human CSF appeared to be positively correlated with leukocyte numbers in the CSF. Additionally, plasma levels of CCL5 were significantly elevated in fatal compared to non-fatal human cases (Winter et al., 2004).

Pathogenesis studies in mice have revealed a critical role for CCR5 during flavivirus infection. In the context of WNV infection, significant differences were observed only within the CNS, with *Ccr5*$^{-/-}$ mice exhibiting increased viral loads, concomitant with reduced accumulation of infiltrating CD4^{+} and CD8^{+} T-cells, NK cells and monocytes/macrophages (Glass et al., 2005). The loss of these cells correlated with 100% mortality in *Ccr5*$^{-/-}$ mice compared to wild type controls, where survival was documented to be ∼35%. Of note, viral clearance from the spleen in both *Ccr5*$^{-/-}$ and wild type mice was identical and underlines

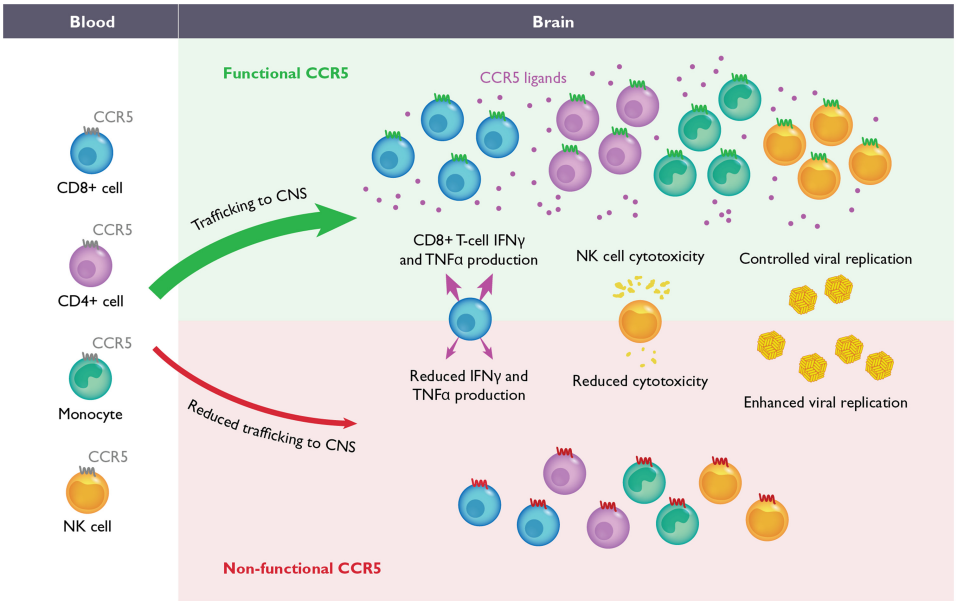

FIGURE 2 | The role of CCR5 during flavivirus-induced encephalitis. During WNV and Japanese encephalitis virus (JEV) infection in mice, the migration of $CD4^+$ T-cells, $CD8^+$ T-cells, NK-cells and monocytes from the blood into CNS is required for efficient control of viral replication and recovery (upper). In the absence of CCR5 in mice, the migration of these leukocytes into the CNS is delayed and/or severely impaired (lower). During JEV infection, the absence of CCR5 also results in inefficient NK and $CD8^+$ T-cell effector functions as well. Although the function of CCR5 has not been evaluated during WNV infection in humans, homozygosity for the complete loss-of-function mutation, *CCR5Δ32*, has been correlated with increased severity of WNV and tick borne encephalitis virus infections. It is anticipated that blockade of CCR5, either in mice or humans, may increase susceptibility to neurotropic flaviviruses.

the importance of a CNS specific role of CCR5 in WNV-infected mice. Adoptive transfer of $CCR5^+$ splenocytes into $Ccr5^{-/-}$ mice restored the survival rate similar to that in wild type infected mice. These data strongly suggest that WNV infection within the CNS triggers a CCR5-dependent influx of leukocytes into the CNS that is required for viral clearance and survival (**Figure 2**).

Similar results, showing a critical role for CCR5 in host defense, have been obtained in a mouse model of JEV infection (Larena et al., 2012). Larena et al. reported that $Ccr5^{-/-}$ mice infected with JEV exhibit increased mortality (64%) compared to wild type mice (28%), correlating with higher viral titers in the CNS. Peripheral control of virus and the induction of the humoral immune response were similar between wild type and $Ccr5^{-/-}$ mice. However, NK and $CD8^+$ T-cell effector functions were blunted in the absence of CCR5 compared to wild type mice (**Figure 2**). Rather than a loss of leukocyte infiltration, as observed in WNV infection, a transient delay in the recruitment of NK cells, $CD4^+$ and $CD8^+$ T-cells, and monocytes into the infected CNS was observed. Together, these data suggest that the functions of CCR5 are critical for controlling both leukocyte trafficking as well as effector functions, which is required for efficient clearance of virus from the CNS.

During SFV infection in mice, evaluation of chemokine transcript numbers in the CNS revealed high induction of CCL3, CCL4, and CCL5 expression, suggesting a possible role for CCR5 in pathogenesis of SFV (Michlmayr et al., 2014). Although $Ccr5^{-/-}$ mice have not been tested, we evaluated the efficacy of a small molecule CCR5 receptor antagonist, named DAPTA, in a mouse model of SFV. DAPTA is a synthetic peptide comprising eight amino acids (185–192) of the gp120 V2 region of HIV-1 that binds competitively to the ligand-binding site of CCR5 (Polianova et al., 2005). Mice were infected with either the avirulent or virulent strain of SFV and then treated subcutaneously once daily with DAPTA, starting on day 3 post infection. The results reveal no significant difference in mortality during virulent SFV infection and no significant reduction of leukocytes entering the brain of treated mice compared to untreated mice (both virulent and avirulent strains; **Table 1**). However, the number of CCR5 expressing cells in the brains of treated mice was significantly reduced compared to untreated mice, confirming the efficacy of DAPTA in blocking CCR5. Our data suggest that other chemokine receptors may be involved in attracting leukocytes into the virally infected brain during SFV infection.

Epidemiological studies

Based on the evidence in mouse models suggesting a critical neuroprotective role for CCR5 during flavivirus infections, several epidemiologic studies have been conducted to evaluate the role of CCR5 in humans, specifically testing for the complete loss-of-function mutation *CCR5Δ32*. This phenotype is commonly found at 10% allele frequency in the US (Zimmerman et al., 1997; Berger et al., 1999). In the context of WNV infection, several initial cohort studies, using patient samples collected from the US epidemic, showed a significant increase in *CCR5Δ32* homozygosity among WNV infected individuals with symptomatic outcome compared

to uninfected controls (Glass et al., 2006; Lim et al., 2008, 2010). Further, the frequency of the *CCR5Δ32* mutation among WNV infected individuals who remained asymptomatic was significantly depressed compared to controls, suggesting that all WNV infected *CCR5Δ32* homozygotes progress to symptomatic disease (Lim et al., 2010). Evaluation of *CCR5Δ32* heterozygotes showed no increased susceptibility, suggesting that partial functionality of CCR5 was sufficient to confer protection (Lim et al., 2010). However, these findings have recently been challenged by two additional studies, which failed to find any association of symptomatic WNV infection and *CCR5Δ32* mutation. Reasons for these discrepant results may be due to differences in study design, cohort size, and/or race composition of the tested cohorts and control populations (Bigham et al., 2011; Loeb et al., 2011). Further studies, using large and well characterized cohorts, in which symptom development is documented, should be conducted.

The frequency of the *CCR5Δ32* mutation has also been evaluated in a cohort of TBEV-infected patients (Kindberg et al., 2008). Genotyping for the *CCR5Δ32* mutation revealed an increased frequency among TBE patients, compared to the aseptic meningoencephalitis and control group. Furthermore, the allele frequency of *CCR5Δ32* correlated with disease severity. Although this study evaluated a small number of patients, these data suggest that the *CCR5Δ32* mutation may be a risk factor for developing severe disease after TBEV infection. In a follow up study by Barkhash et al. (2013) using a larger cohort, the authors found no significant association between *CCR5Δ32* mutation and predisposition to TBE.

Among the encephalitic flaviviruses, JEV is responsible for the most cases of encephalitis worldwide (Campbell et al., 2011). Although CCR5 is a critical host factor during JEV infection in mice, an evaluation of *CCR5Δ32* in patients would be difficult in cohorts of JEV-infected individuals, since endemic areas are expected to have a low or absent *CCR5Δ32* allele frequency (Zimmerman et al., 1997; Berger et al., 1999). However, there are several other CCR5 polymorphisms described in the literature that strongly modulate CCR5 expression, and genotype:phenotype association studies using these genetic probes are feasible (Carrington et al., 1999).

CCR5 receptor antagonists

Studies in mice, along with epidemiologic data, suggest that CCR5 is protective in WNV, JEV, and TBEV infection, which highlights the concern for chronic use of CCR5 antagonists in humans. Maraviroc is an FDA-approved CCR5 antagonist that is currently approved for the treatment of HIV-infected patients. Further, this antagonist is being considered for several other inflammatory diseases, with anticipated long-term use (Velasco-Velazquez et al., 2012; Wilkin and Gulick, 2012; Cipriani et al., 2013; Ochoa-Callejero et al., 2013). Based on epidemiologic data showing no change in susceptibility among *CCR5Δ32* heterozygotes, the risk of CCR5 blockade will depend on the amount of CCR5 coverage achieved by the drug. We would anticipate that cessation of CCR5 blockade should fully restore the functionality of this chemokine receptor, in the event that a patient prescribed CCR5 blockers develops symptoms associated with flavivirus infection. More studies will be required to fully understand the impact of CCR5 antagonists during flavivirus infection in humans.

CXCR2

Neutrophils are an important cellular component of the innate immune response and appear to have a role in the pathogenesis of flavivirus infection of the CNS. During WNV encephalitis in mice, neutrophils comprise a significant proportion of the cellular infiltrate within the CNS (Lim et al., 2011). Likewise in humans, neutrophils were predominant in the CSF of patients with neuroinvasive disease (Rawal et al., 2006; Tyler et al., 2006). Like monocytes, neutrophils reside in the bone marrow, and their mobilization is regulated, in part by CXCR2, a chemokine receptor highly expressed on mouse and human neutrophils (Eash et al., 2010). In mice, CXCR2 binds CXCL1 and CXCL2, while in humans, this receptor binds most potently to CXCL8 (Murphy, 1997). A study by Bai et al. (2010) demonstrated that neutrophils play a dual role during WNV infection. Using a Gr-1 or Ly6G neutralizing antibody, the authors showed that depletion of neutrophils prior to infection resulted in reduced viral loads and enhanced survival compared to untreated mice. However, if neutrophils are depleted 1 or 2 days after infection, greater viremia and mortality was observed. These data suggest that, depending on the timing of depletion, neutrophils can be either beneficial and contribute to viral clearance or can be detrimental and increase viral dissemination of WNV in mice. The authors also evaluated the mortality rate between WNV-infected wild type and $Cxcr2^{-/-}$ mice, which revealed a significant increase in survival time in the absence of CXCR2 (Bai et al., 2010). Viremia was decreased on day 1 in $Cxcr2^{-/-}$ mice, compared to wild type mice; however, by day 3, viremia was higher in CXCR2 deficient mice. These data suggest that CXCR2 is involved in early migration steps that affect viral dissemination but may contribute to viral clearance during the later phases of infection. Unfortunately, the migration of neutrophils into the CNS was not measured in the absence of CXCR2, and more studies are required to understand the chemokine receptors required for their entry into the CNS and their function.

No mouse studies have addressed the role of neutrophils during JEV infection. However, several lines of evidence suggest that these cells are important *in vivo*. Firstly, JEV has been shown to induce neutrophilia in mice infected intraperitoneally; this has also been observed in human infections as well (Chaturvedi et al., 1979; Johnson et al., 1986; Mathur et al., 1988, 1992; Chung et al., 2007). Secondly, significant induction of CXCL8 levels was concomitant with the presence of neutrophils in the CSF of patients positive for JEV (Winter et al., 2004). Notably, significantly higher levels of CXCL8 were associated with patient mortality (Singh et al., 2000). Studies determining the signals involved in neutrophilia versus monocytosis, and how these are differentially regulated during JEV and WNV infection, could provide useful insights into the different clinical presentations observed in patients.

Murray Valley encephalitis virus (MVEV) is a mosquito-transmitted neurotropic flavivirus, endemic to parts of Australia and Papua New Guinea. Using a mouse model of MVEV infection, one study showed that the leukocytic infiltrate in the CNS

predominantly comprised neutrophils, which appeared to colocalize with neurons and was preceded by the induction of CXCL1 expression (Andrews et al., 1999). Depletion of neutrophils, using a Gr-1 antibody, resulted in increased survival (55%) compared to isotype control-treated mice, where infection was uniformly lethal. These data suggest that the production of iNOS in the CNS may be the mechanism by which neutrophils are promoting pathogenesis in this model. No further studies were conducted to understand the temporal and organ-specific roles of neutrophils in promoting viral replication or to determine which receptor is utilized for tissue migration.

In the context of SFV infection, we showed that neutrophil-associated chemokines CXCL1 and CXCL2 were not expressed in the brain of mice infected with the avirulent strain. Consistent with this, no neutrophils were observed in the CNS as assessed by flow cytometric analysis and immunohistochemistry (Michlmayr et al., 2014). In contrast to this, CXCL2 transcripts were highly upregulated in murine brains infected with the virulent strain of SFV, exhibiting a >2000-fold increase in CXCL2 transcripts compared to healthy control brains. Neutrophils were detected in the CNS of virulent SFV-infected mice by histology, and the extent of CXCL2 upregulation correlated with disease severity, as lethally SFV-infected mice displayed higher CXCL2 induction in the CNS compared to asymptomatic mice (Michlmayr et al., 2014). The function of neutrophils in this model is unclear, and more studies are needed to understand their role, if any, in SFV and other neurotropic alphavirus infections.

CXCR2 receptor antagonists

Because of the dominant role of CXCL8 in the activation and recruitment of neutrophils in humans, several antagonists for its receptors CXCR1 and CXCR2 have been developed for a wide range of diseases, including COPD, cystic fibrosis, and pancreatic islet transplantation (Horuk, 2009; Bachelerie et al., 2013). Reparixin, a non-competitive allosteric inhibitor of CXCR1 and CXCR2, appears to be the farthest along, having now entered clinical phase III trials in Europe and the United States (Citro et al., 2012). Several others are also in the pipeline, including ones specific for CXCR2 (Dwyer et al., 2006; Chapman et al., 2007; Gonsiorek et al., 2007). The function of neutrophils during flavivirus infection, both in the periphery and CNS, is unclear. However, since depletion of neutrophils prior to infection promotes survival, individuals chronically administered CXCR1/2 antagonists may experience some level of protection during WNV infection. In the rare event that an individual is administered a CXCR1/2 blocker soon after infection, increased WNV replication in the periphery could result in a more aggressive disease outcome.

CXCR3

The chemokine receptor CXCR3 is found at high levels on activated T-cells and NK-cells, and it can bind to chemokine ligands CXCL9, CXCL10, or CXCL11. The induction of these ligands is nearly always associated with cell-mediated immunity, and these chemokines are considered to be interferon-stimulated genes. The recruitment of antigen-specific T-cells is a critical step in viral clearance within the CNS, and the CXCL10:CXCR3 axis appears to be particularly important in this process, at least in the context of WNV (**Figure 3**). CXCL10 is the most highly induced chemokine in the CNS in mouse models of WNV, JEV, TBEV, and SFV encephalitis (Klein et al., 2004; Glass et al., 2005; Garcia-Tapia et al., 2007; Cao et al., 2011; Yang et al., 2011; Palus et al., 2013; Michlmayr et al., 2014). In addition, the other CXCR3 ligands, CXCL9 and CXCL11, are also often upregulated in the infected CNS, although no studies have evaluated the role of these chemokines *in vivo* (Klein et al., 2004; Glass et al., 2005; Michlmayr et al., 2014).

The most thorough analyses of CXCL10 and CXCR3 come from data collected for WNV infection. In WNV-infected blood donors, CXCL10 is significantly upregulated in the plasma during the viremic phase of infection, with levels significantly decreased after seroconversion, consistent with its role as an interferon-stimulated gene (Tobler et al., 2008). Although *in vitro* studies using primary brain cell cultures, showed CXCL10 expression in WNV-infected astrocytes and microglia, the production of CXCL10 *in vivo* appears to be primarily by infected neurons (Cheeran et al., 2003; Shirato et al., 2004; Klein et al., 2005). Using CXCL10-deficient mice and an anti-CXCL10 neutralizing antibody, Klein et al. (2005) observed a significant reduction in $CD8^+$ T-cells within the CNS, higher viral titers in the brain, and enhanced mortality compared with wild type mice (**Table 1**). These results were phenocopied using *Cxcr3*$^{-/-}$ mice (Zhang et al., 2008). Of note, the number of $CD4^+$ T-cells was not significantly reduced in the WNV-infected brain, suggesting that CXCL10 is important for the specific recruitment of $CD8^+$ T-cells and that other receptors may compensate for the recruitment of $CD4^+$ T-cells (**Figure 3**). Additionally, the effect of CXCL10 on the pathogenesis of WNV infection appears to be CNS-specific since clearance of WNV in peripheral tissues were identical compared to wild type infected mice (Klein et al., 2005). Thus, these data show an indispensable role for CXCL10 in the recruitment of CXCR3-expressing $CD8^+$ T-cells into the CNS.

In addition to the role of the CXCL10:CXCR3 axis in regulating $CD8^+$ T cell migration into the CNS, a more recent study has demonstrated a role for this chemokine:receptor pair in promoting neuronal apoptosis during WNV encephalitis. The authors found that TNF-α produced during WNV encephalitis caused specific downregulation of CXCR3 expression on infected and bystander neurons. Downregulation of CXCR3 through pretreatment with TNF-α or in *Cxcr3*$^{-/-}$ mice resulted in increased neuronal survival through delayed activation of caspase 3. These data suggest that although CXCL10:CXCR3 signaling is protective in its capacity to promote effector $CD8^+$ T-cell migration to assist with viral clearance, the same signaling event on neurons may induce neuronal apoptosis and exacerbate immune-mediated damage (**Figure 3**). These data exemplify the complexity of chemokines during CNS inflammation and suggest that the use of CXCR3-specific antagonists may be beneficial or detrimental, depending on cell-type specific effects and timing.

In TBEV-infected patients presenting with neuroinvasive disease, two studies reported high levels of CXCL10 in the CSF (Lepej et al., 2007; Zajkowska et al., 2011); cytoanalysis of CSF samples revealed that the majority of $CD4^+$ T-cells were positive for CXCR3. These data suggest that the CXCL10:CXCR3 axis

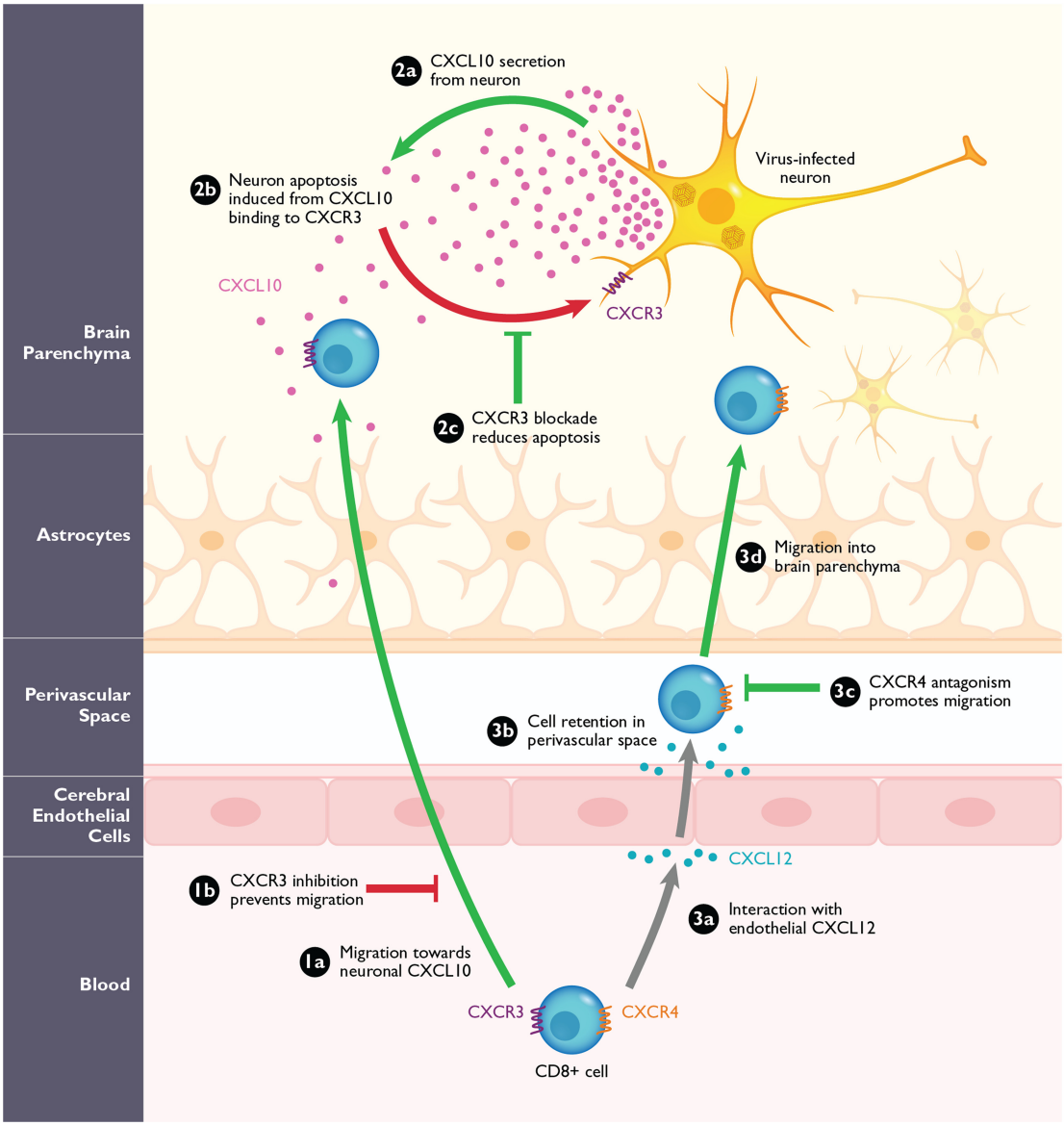

FIGURE 3 | The role of CXCR3 and CXCR4 during arbovirus-induced encephalitis in mice. (1a) $CXCR3^+CD8^+$ T-cells migrate into the CNS in response to high levels of neuronal CXCL10. Loss of CXCR3 or its ligand CXCL10, or antagonizing this interaction **(1b)** prevents the migration of $CD8^+$ T-cells into the CNS during WNV in mice. **(2a)** Virally infected neurons are the predominant source of CXCL10 in the CNS during WNV infection. **(2b)** Neuronal CXCL10 can engage CXCR3 that is upregulated on infected neurons and induce apoptosis. **(2c)** Blockade of CXCR3 with antagonists or CXCL10 neutralizing antibodies leads to the reduced binding of neuronal CXCL10 to neuronal CXCR3 and result in reduction of apoptosis and increased neuronal survival. **(3a)** During WNV infection, $CXCR4^+CD8^+$ T-cells migrate toward endothelial CXCL12, expressed on the inflamed cerebral endothelium. **(3b)** The interaction of CXCL12 and CXCR4 causes the $CD8^+$ T-cells to be retained within the perivascular space. **(3c,d)** After blockade with the CXCR4 antagonist, AMD3100, the $CD8^+$ T-cells are released and can migrate into the brain parenchyma where they promote clearance of WNV. Pathological steps are depicted in red; beneficial steps involved in increasing survival and improving disease outcome in the host are depicted in green. Gray arrows signify functions that are neither beneficial nor pathogenic.

may also be critical for T-cell migration into the CNS. Although CXCL10 expression is highly upregulated in the CNS during TBEV infection in mice, the specific role of these cells in directing T-cell migration into the CNS has not been studied (Tigabu et al., 2010; Palus et al., 2013).

In the context of alphavirus infection, the role of CXCR3 has not been evaluated yet. CXCL9 and CXCL10 are highly upregulated in the CNS of SINV-infected mice, and preceded the infiltration of T-cells and B-cells (Metcalf et al., 2013). Flow cytometric analyses of B-cells within the SINV infected CNS revealed high and uniform expression of CXCR3 on these cells. During SFV infection with both virulent and avirulent strains, CXCL9 and CXCL10 were highly upregulated in the CNS of infected mice as well, correlating with a large influx of T-cells, primarily $CD8^+$ T-cells, into the CNS

(Michlmayr et al., 2014). To evaluate the role of CXCR3 in the context of SFV infection, we employed a small molecule antagonist, compound 21, an imidazo-pyrazine derivative, which has been shown to block binding of CXCL9, CXCL10, and CXCL11, and is specific for CXCR3 (Du et al., 2009). SFV-infected mice were treated subcutaneously once daily starting on day 3 post infection. Treatment of avirulently infected mice with compound 21 resulted in a significant decrease of T-cell infiltration into the CNS compared to untreated mice, suggesting that CXCR3 is involved in the T-cell migration into the inflamed brain (**Figure 3**). Surprisingly, blockade of CXCR3 during lethal SFV infection did not result in a change in survival (**Table 1**). However, the combined blockade of CXCR3 and CCR2, did result in significantly enhanced survival compared to untreated mice (**Table 1**). Thus, the dual blockade of CXCR3 and CCR2 is necessary to achieve a survival benefit during lethal SFV-induced encephalitis. It is likely that these antagonists are inhibiting both T-cells and monocytes, implying that lethality in this model is immune-mediated. Furthermore, our data suggest that dual blockade of chemokine receptors may be more effective for treating diseases, a paradigm known as polypharmacology (Roth et al., 2004; Frantz, 2005; Overington et al., 2006). This concept is reviewed in more detail elsewhere (Overington et al., 2006; Horuk, 2009).

CXCR3 receptor antagonists

Compound 21, synthesized by Amgen, has been shown to bind to CXCR3 with high potency, inhibiting the binding of cognate CXCR3 ligands (Du et al., 2009). In addition to this compound, there are several other CXCR3 blockers in clinical development for the treatment of rheumatoid arthritis and psoriasis and is extensively reviewed elsewhere (Bachelerie et al., 2013; Michlmayr and McKimmie, 2014). At least during WNV infection, there appears to be a dual function of the CXCR3:CXCL10 axis; firstly, for the recruitment of antiviral $CD8^+$ T-cells into the CNS, and secondly, to promote neuronal apoptosis (Klein et al., 2005; Bhowmick et al., 2007; Zhang et al., 2008). Despite this, the effect of global CXCR3 and CXCL10 deficiency resulted in increased mortality, suggesting that the dominant protective function of CXCR3 is at the level of $CD8^+$ T-cell migration into the CNS, which strongly outweighs its role in promoting neuronal apoptosis (Zhang et al., 2008, 2010). Thus, we anticipate that the blockade of CXCR3 during flavivirus induced encephalitis may promote pathogenesis. In addition to CXCR3 antagonists, a neutralizing CXCL10 antibody, MDX-1100, is also being tested by Medarex for the treatment of rheumatoid arthritis and is currently in clinical phase II studies (Yellin et al., 2012). Similar to CXCR3 antagonism, MDX-1100 may block effector T-cell migration into the CNS and increase pathogenesis.

CXCR4

CXCR4 and its sole ligand CXCL12 are among the most highly conserved in the chemokine superfamily (Lee et al., 1999; Zlotnik et al., 2006). CXCR4 has multiple critical functions, including embryonic development, homeostasis, and lymphoid organ retention as well as in serving as a coreceptor for HIV-1. As a result of its role in HIV entry, a small molecule inhibitor, AMD3100, was identified as a potent and selective antagonist for CXCR4. Under the name Plerixafor or Mozobil, AMD3100 is now FDA-approved and used to mobilize stem cells in non-Hodgkin lymphoma and multiple myeloma patients.

The role of CXCL12:CXCR4 during CNS inflammation in flavivirus and alphavirus infections is not well characterized. Because of the known role of the CXCL12:CXCR4 axis in cellular retention within the bone marrow, McCandless et al. (2008) hypothesized that the interaction of CXCL12 could be an important retention signal for cells migrating into the CNS. Indeed, $CD8^+$ T-cells are restricted at the BBB through interactions with endothelial CXCL12 (**Figure 3**). Interrupting this interaction through the continuous administration of AMD3100 from the initial time of WNV infection in mice, resulted in the release of $CD8^+$ T-cells in the perivascular space, allowing subsequent migration of these cells into the brain parenchyma, and is leading to enhanced viral clearance and survival (**Figure 3**; **Table 1**). Importantly, the authors showed the same pattern of perivascular retention of T-cells through CXCL12 and CXCR4 expression patterns in WNV-infected patients with neuroinvasive disease (McCandless et al., 2008). The authors also showed that glial cell activation was decreased in AMD3100-treated mice that can subsequently minimize pathological immune activation within the CNS. These data are even more impressive and relevant, since AMD3100 would be expected to function similarly in infected humans due to high conservation of CXCR4 between mice and humans.

CXCR4 receptor antagonists

Since AMD3100 is already FDA-approved (Mozobil, Plerixafor), the use of this antagonist for the treatment of WNV-infected individuals is possible. In fact, McCandless et al. (2008) have demonstrated that treatment of WNV-infected mice with AMD3100, starting on day 4 post infection, led to a prolonged survival, although no overall survival benefit was observed. These data are still very encouraging since increasing survival time, along with supportive care or in combination with other therapeutics as they become available, may provide important therapeutic options. In mice and humans, AMD3100 is capable of increasing overall numbers of leukocytes in the blood, which may also contribute to enhanced survival (Capoccia et al., 2006; McDermott et al., 2011).

CONCLUSION

The emergence and spread of arboviral infections in the past few decades has highlighted the unpredictable nature of human outbreaks and emphasizes the need for novel treatment and prevention measures. The recent outbreak of WNV disease in the United States, and its continued emergence in several regions within Europe are just a few examples (Centers for Disease Control and Prevention [CDC], 2013; Sambri et al., 2013). Although there are vaccines available for the prevention of JEV and TBEV infection, no vaccines exist for human WNV infection, and no specific therapeutics are currently available for the treatment of neuroinvasive diseases caused by any arbovirus. Therefore, there is an urgent need for novel intervention strategies, either in the form of antivirals or immunomodulators that can block viral replication, boost protective immune responses, and minimize CNS injury. Furthermore, these therapeutics should be efficacious after the onset of symptoms, when the virus has entered the CNS and neuroinvasive symptoms have developed in patients.

Leukocyte migration is critically important during all phases of viral replication *in vivo*, and it is apparent that the chemokine network plays an integral role in the generation of an effective host immune response in the CNS. It is clear that both innate and adaptive immune responses are required for responding to and counteracting viral replication and spread, and since naturally acquired arboviral infections are initiated in the periphery, the timing and magnitude of both innate, as well as T- and B-cell responses, are critical for efficient viral control once the virus has accessed the CNS (Samuel and Diamond, 2005; Diamond and Gale, 2012). However, viral infections within the CNS require a response that is rapid and effective, but is not excessively exuberant as this could cause collateral damage to functionally critical and non-renewable cell types. A suboptimal or excessive immune response, or insufficient timing of immune responses, could alter the outcome of arboviral infections. Studies investigating chemokine-mediated leukocyte trafficking as well as other non-trafficking related functions during arboviral infections have provided great insight into our understanding of viral pathogenesis. Despite the great redundancy in the system, critical and non-overlapping functions for specific chemokines and receptors have been identified. Indeed, the migration of cells from the blood into the CNS, activation of effector cell function, mobilization of leukocytes, and retention of cells in the perivascular space are just a few examples. Thus, manipulating chemokine receptors therapeutically is a particularly attractive means by which to modulate outcome of infection. However, it is important to note that nearly all of the data so far have been evaluated using knock-out systems in mice; thus, our knowledge of the role of chemokine receptors on specific subsets of cells is incomplete primarily due to the lack of conditional knock out systems for most chemokine receptors. As reagents become available, it is imperative to further our understanding of how each receptor functions in a cell-type and organ-specific manner. Furthermore, chemokine receptor antagonists may be more relevant to understand the effect of therapeutics on disease outcome compared to knock-out model systems.

There are many areas of research that have remained relatively unexplored with regards to arboviral encephalitides: What are the key chemokine-mediated events that take place in local draining lymph node? What receptors coordinate optimal B- and T-cell activation within lymphoid tissues? How do neutrophils mobilize and migrate into the CNS? Still many questions remain, and there are many cell types that have not been studied in the context of arboviral encephalitis, most notably microglia. Several studies have shown that microglia are highly activated and proliferate during WNV encephalitis, and studies *in vitro* have found that these cells respond to virus through the TLR-3 pathway (Glass et al., 2005; Town et al., 2006; Getts et al., 2008). However, the function of microglia during WNV encephalitis *in vivo* is currently unknown. Since these cells exclusively express CX_3CR1 in the healthy CNS, and its ligand CX_3CL1 is constitutively expressed by neurons, this receptor:ligand interaction is likely to function during infection (Harrison et al., 1998; Cardona et al., 2006; Combadiere et al., 2007). In a healthy brain, the interaction between CX_3CL1:CX_3CR1 is hypothesized to suppress certain aspects of microglial activity (Zujovic et al., 2000; Cardona et al., 2006). Based on studies in numerous other models, loss of the signal, as in the case of CX_3CR1-deficiency, results in microglia displaying an increased propensity for activation and function (Liu et al., 1998; Soriano et al., 2002; Sunnemark et al., 2005; Cardona et al., 2006; Fuhrmann et al., 2010). How the loss of CX_3CR1 expression on microglia alters the outcome of WNV and other arboviral infections of the CNS will provide critical insights into their role in neuropathogenesis.

The use of chemokine receptor antagonists is an active area for drug development but is complicated by their pleiotropic functions that may have opposing effects. One such example is the dual role of CXCR3 during WNV pathogenesis. It has been shown that CXCR3 is critical for $CD8^+$ T-cell migration into the CNS and promotes viral clearance and survival. However, it has also been shown that neuronally expressed CXCR3, and its interaction with CXCL10 on WNV-infected neurons, promotes neuronal apoptosis. Thus, CXCR3 antagonism would have both a protective and pathogenic effect on disease outcome; the diametric consequences of CXCR3 blockade could result in unanticipated effects. Additionally, due to the positive and negative pressures that contribute to the evolution of chemokine receptors over time, the role of any given receptor could be beneficial in one setting and detrimental in another. This complexity is best illustrated with CCR5, which functions in promoting infection in the context of HIV-1 infection but has the reciprocal effect during WNV infection (Lim et al., 2006). Thus, CCR5 antagonism, using FDA-approved Maraviroc for the treatment of HIV-infected individuals, carries the cost of promoting symptomatic WNV disease.

It is pivotal to understand the underlying immunological mechanism of encephalitis in order to develop effective treatment of acute viral encephalitis. New insights into the role of chemokines and their receptors in these contexts are also informative for studies of brain inflammation caused by multiple sclerosis, Alzheimer's or Parkinson's disease. The successful development of CCR5 and CXCR4 antagonists in humans demonstrates that chemokine receptors are feasible and effective targets that have the capacity to modulate disease. In fact, studies in mice and humans predict that these two antagonists would have opposite effects in human WNV disease, with Maraviroc promoting symptomatic disease and CXCR4 blockers promoting survival during infection. Because most chemokine receptor antagonists currently being developed will likely be administered chronically, it is critical to understand how these therapies may affect the individual in the context of their specific infection. This is an important goal that has been and should continue to be tested in the laboratory setting. Moreover, it is important to note that data obtained in inbred mouse models may not be applicable to humans. Thus, treatment of infected individuals with chemokine receptor antagonists may not function as anticipated based on mouse studies and should be approached with caution. Despite the success of Maraviroc and Mozobil/Plerixafor, there have been many receptor antagonists that have failed in clinical trials. Due to the redundancy of the chemokine system, antagonists that inhibit more than one receptor or the use of several compounds in conjunction may prove beneficial.

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Neuronal CC chemokines: the distinct roles of CCL21 and CCL2 in neuropathic pain

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The development of neuropathic pain in response to peripheral nerve lesion for a large part depends on microglia located at the dorsal horn of the spinal cord. Thus the injured nerve initiates a response of microglia, which represents the start of a cascade of events that leads to neuropathic pain development. For long it remained obscure how a nerve injury in the periphery would initiate a microglia response in the dorsal horn of the spinal cord. Recently, two chemokines have been suggested as potential factors that mediate the communication between injured neurons and microglia namely CCL2 and CCL21. This assumption is based on the following findings. Both chemokines are not found in healthy neurons, but are expressed in response to neuronal injury. In injured dorsal root ganglion cells CCL2 and CCL21 are expressed in vesicles in the soma and transported through the axons of the dorsal root into the dorsal horn of the spinal cord. Finally, microglia *in vitro* are known to respond to CCL2 and CCL21. Whereas the microglial chemokine receptor involved in CCL21-induced neuropathic pain is not yet defined the situation concerning the receptors for CCL2 in microglia *in vivo* is even less clear. Recent results obtained in transgenic animals clearly show that microglia *in vivo* do not express CCR2 but that peripheral myeloid cells and neurons do. This suggests that CCL2 expressed by injured dorsal root neurons does not act as neuron-microglia signal in contrast to CCL21. Instead, CCL2 in the injured dorsal root ganglia (DRG) may act as autocrine or paracrine signal and may stimulate first or second order neurons in the pain cascade and/or attract CCR2-expressing peripheral monocytes/macrophages to the spinal cord.

Keywords: neuropathic pain, microglia reaction, chemokines, neuron-microglia signaling, DRG neurons, LDV vesicles, regulated release pathway

THE IMPORTANCE OF PAIN

An important aspect for the survival of all organisms is the sensation of potential harmful (noxious) threats, which often are experienced as pain (nociception). Accordingly, it has been known for a long time that, even humans with congenital insensitivity to pain often die as children because they fail to notice injuries and illnesses, which underlies the importance of proper nociception (see for review: Indo, 2001; Cox et al., 2006; Costigan et al., 2009). Nociceptive neurons, like all primary afferent neurons, innervate organs and the periphery. Their cell bodies are located in the dorsal root ganglia (DRG) meaning that these neurons reside outside of the central nervous system. There are two main types of nociceptive neurons, unmyelinated C fibers and thin myelinated Aδ fibers, that both mainly express so called transient receptor potential (TRP) channels in order to respond to intense mechanical or thermal stimuli (see for review: Dhaka et al., 2006; Szallasi et al., 2007). Nociceptive neurons project to the dorsal horn of the spinal cord (mainly to Lamina I and II) where they signal to second-order neurons that project to higher pain centers in hypothalamus and cortex. The nociceptive signal in the dorsal horn of the spinal cord is also transmitted to interneurons that are important for the fast nociceptive withdrawal reflex. The physiologic nociceptive signal occurs in response to acute stimuli and continues only in its presence; meaning that physiologically nociceptive pain is rather short lived.

INFLAMMATORY PAIN

When tissue damage is more severe and causing a subsequent inflammatory reaction, nociception is prolonged and sensitized, thus the pain sensing system of the injured body parts undergoes profound changes in its responsiveness (Scholz and Woolf, 2007; Latremoliere and Woolf, 2009; Ren and Dubner, 2010; Johnson et al., 2013). As a result of this pain hypersensitivity the affected body parts are protected from further physical contact, which is to aid the healing process. This type of pain or hypersensitivity is directly caused by local inflammation in the injured or infected body parts and is therefore called inflammatory pain. In fact one of the hallmarks of inflammation in general is pain.

There are several ways by which nociception is sensitized by inflammation. Inflammatory mediators might directly affect TRP channel activity. Several compounds of the "inflammatory soup" such as bradykinins, prostaglandins, leukotriene B4 and many

others are known to sensitize TRPV1 activity (Szallasi et al., 2007). Furthermore, it is known that pro-inflammatory cytokines including IL-1β or TNFα also directly affect the signaling and excitability of sensory neurons (see for review: Uçeyler et al., 2009). Moreover, it has been shown that these pro-inflammatory cytokines induce the release of several neuropeptides, such as substance P (SP) or calcitonine gene-related peptide (CGRP) from C fibers, which in turn initiate a higher expression of pain sensing receptors and increased excitability in sensory neurons; a process called neurogenic inflammation (Uçeyler et al., 2009). Thus, the impact of inflammatory factors on the pain sensing system is manifold and yet by far not completely understood. The fact that injection of almost all known pro-inflammatory factors can cause temporary pain or pain hypersensitivity shows the robustness of this tight connection between inflammation and pain sensation. Being in aid of the healing process, inflammatory pain persists until the end of the repair process, it disappears when inflammation is over. Thus, although inflammatory pain may last for several weeks, it is generally temporary and thus reversible.

THE DARK SIDE OF NOCICEPTION: NEUROPATHIC PAIN

Physiological pain is generally connected to pathology and in aid of the organism. However, sometimes pain itself becomes the primary clinical problem, meaning that pathological pain neither protects nor supports healing. Pathological pain occurs when nociceptive thresholds are reduced such that normally innocuous stimuli become painful (allodynia) or when pain is sensed even in the absence of a given stimulus. These phenomena are called neuropathic pain and are due to changes higher up in the pain cascade (spinal cord or brain stem), which are summarized as central sensitization (Latremoliere and Woolf, 2009). Central sensitization is characterized by reduced inhibition and increased neuronal excitability/synaptic efficacy of the neurons of the nociceptive pathway, which as a result uncouples pain sensation from noxious stimuli (Latremoliere and Woolf, 2009).

Neuropathic pain is a consequence of damage of peripheral nerves possibly caused by mechanical trauma, metabolic disorders (diabetes), neurotoxic chemicals, infections or tumors (Dworkin et al., 2003). Neuropathic pain treatment has conventionally been applied on the basis of the underlying disease, which means that it was anticipated that treatment of the disease would resolve the pain symptoms (Dworkin et al., 2007). However, since the primary disease and the resulting peripheral nerve damage only initiates the cascade that subsequently leads to development and maintenance of neuropathic pain, such an etiological approach does not capture the essential feature of neuropathic pain; central sensitization. As a consequence potential treatments for neuropathic pain should prevent, inhibit or reverse the various mechanisms occurring in central sensitization (Latremoliere and Woolf, 2009).

Nerve damage surely causes an inflammatory reaction at the lesion site, which is why neuropathic pain shares many features with inflammatory pain. However, in contrast to inflammatory pain it is the nerve injury itself with its profound impact that most likely initiates central sensitization. For example, comparing the changes in gene expression in the DRG neurons in animals after induction of inflammatory pain (complete freund's adjuvant (CFA) injection) or nerve injury (chronic constriction injury (CCI) model) revealed by far more changes in mRNA expression in the latter paradigm, where hundreds of genes (approximately 5% of all detected genes) were affected by the nerve injury (Costigan et al., 2002; Rodriguez Parkitna et al., 2006). These changes were probable due to the loss of trophic support from the target organ and/or caused by the various signals that are released at the site of injury. The most prominent changes in mRNA expression were attributed to the following functional classes: transcription and translation, cellular metabolism, cytoskeleton, neurotransmission and inflammation (Costigan et al., 2002). Those changes are most likely linked to survival and re-grow of the injured neurons, but also affect their sensitivity and signaling capacities.

CENTRAL SENSITIZATION

The injured peripheral neurons with their cell bodies in the DRGs are not the only neurons of the pain axis that respond to nerve injury. Electrophysiological changes in second order neurons that project from lamina I and II of the dorsal horn to the brain are characteristic for central sensitization and thus important for the development of neuropathic pain. There is evidence that the down-regulation of the potassium-chloride transporter 2 (KCC2) in lamina I neurons, in response to peripheral nerve injury is leading to an alteration in the chloride equilibrium of those cells. This altered chloride equilibrium attenuates GABAergic inhibitory synaptic transmission, or may even switch GABAergic signals from inhibitory to excitatory (Coull et al., 2005). In lamina II, neurons cause peripheral nerve injury an increase in synaptic drive to excitatory neurons, whereas the opposite is the case for inhibitory neurons in lamina II (Biggs et al., 2010). Thus, peripheral nerve injury leads to a substantial state of disinhibition, due to loss of GABAergic inhibition and a reduction in glycinergic inhibitory signaling, which, in combination with a strengthened excitatory signaling is essential for neuropathic pain (Latremoliere and Woolf, 2009). These changes in dorsal horn neurons show that peripheral nerve damage is "recognized" in more central brain parts. Indeed various mRNA expression profiling experiments show that peripheral nerve injury not only affects the cell bodies of the injured nerve in the DRG (Costigan et al., 2002; Rodriguez Parkitna et al., 2006), but also leads to profound changes in the mRNA expression in the ipsilateral dorsal horn of the spinal cord (Griffin et al., 2007). Depending on the used peripheral nerve damage model these changes varied considerably, both qualitatively and quantitatively. After spared nerve injury (SNI) 184 mRNA transcripts were found changed in the spinal cord, 310 changes in the mRNA expression pattern were found in response to CCI and after spinal nerve ligation (SNL) 399 mRNA changes were observed (Griffin et al., 2007). All models have their own specific characteristics, which are for example reflected by the differences in the death rate of DRG neurons (see for review: Costigan et al., 2009) and may explain the differences in gene expression. However, all these different types of injury lead to neuropathic pain in animal models indicating that those 54 mRNAs that were shared by all three models might be important for central sensitization and neuropathic pain (Griffin et al., 2007). Interestingly, the largest functional group

out of those 54 was associated with immune function (Griffin et al., 2007).

It has been recognized in the last decade that multiple immunological processes are participating in neuropathic pain phenomena. Peripheral nerve injury leads to an inflammatory reaction directly at the site of the injured nerve and of the DRGs, where an early and prominent infiltration of peripheral macrophages is found observed (see for review: Scholz and Woolf, 2007). Given the importance of central sensitization in neuropathic pain, however, it is required to understand the changes in the dorsal horn of the spinal cord. Here the situation with respect to peripheral macrophages is less clear. It was reported that an early and prominent infiltration by peripheral macrophages does not occur in the spinal cord; moreover, a depletion of peripheral macrophages did not affect the development of neuropathic pain (Rutkowski et al., 2000; Mitchell et al., 2008, ref 100 from Ren and Dubner). In agreement with these findings, it was shown that the blood-spinal cord barrier of the spinal cord is not greatly affected after spinal nerve injury (Abram et al., 2006; Lu et al., 2009; Calvo et al., 2010). On the other hand Zhang and co-workers described that, in response to peripheral nerve injury macrophages invade the spinal cord, where they subsequently differentiate into microglia-like cells (Zhang et al., 2007). Moreover, it was shown in another study that spinal nerve injury led to a rapid and transient opening of the blood-spinal cord barrier (Beggs et al., 2010). Thus, whether or not peripheral myeloid cells invade the spinal cord in response to peripheral nerve injury is an unresolved issue at the moment. Irrespective of these conflicting results it is widely believed that the first cellular reaction in response to peripheral nerve injury is a rapid change in microglia morphology and physiology (see for recent review: McMahon and Malcangio, 2009).

MICROGLIA

Microglia are the primary immune cells of the CNS parenchyma that are derived from mesoderm as they stem from very early myeloid cells (microglia precursors) that in the mouse at around embryonic day 8–9 invade the developing nervous tissue (see for review: Prinz and Mildner, 2011). Due to their origin microglia share many features with peripheral myeloid cells, but they also show brain specific properties (Ransohoff and Cardona, 2010; Prinz and Mildner, 2011). In the adult brain and spinal cord microglia are more or less evenly distributed, and it is undisputed that these cells are the first line of defence which are activated upon any type of brain injury (Kreutzberg, 1996; Streit, 2002; van Rossum and Hanisch, 2004; Hanisch and Kettenmann, 2007; Biber et al., 2006). Microglia have small cell bodies, fine, long and heavily branched (ramified) processes that claim a territory which does not overlap with the territory of neighboring microglia. Life cell imaging studies using two-photon microscopy have shown that microglia rapidly move those processes in the non-challenged brain thereby palpating their direct environment, making them very active "surveillant" cells, rather than "resting" as long been thought (Nimmerjahn et al., 2005; Ransohoff and Cardona, 2010). In line with this "surveillance" function it was observed that microglia respond to cell damage rapidly within several minutes (Nimmerjahn et al., 2005) with changes in their morphology that follow a stereotypic pattern (Kreutzberg, 1996; Streit, 2002). Since these morphological changes are stereotypic and occur irrespective of the type of insult, the term "activated microglia" became misleading over the years, because it suggests a single functional state of those cells, which is known now not to be true (Hanisch and Kettenmann, 2007; Ransohoff and Cardona, 2010). It is now clear that microglia respond with a variety of different reactions by integrating multifarious inputs (Schwartz et al., 2006; Biber et al., 2007; Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009; Ransohoff and Cardona, 2010). It is therefore concluded that general terms like "microglia activation" or "activated microglia" are not sufficient to depict the function of microglia. Instead the different functional states of microglia should be described with respect to a given physiological or pathological situation (McMahon and Malcangio, 2009; Biber et al., 2014).

MICROGLIA IN NEUROPATHIC PAIN

Approximately two decades ago it was recognized that dorsal horn microglia respond to peripheral nerve injury with a morphological change and up-regulation of several microglial markers (Eriksson et al., 1993). These findings, together with early observations that inflammatory mediators are involved in neuropathic pain (Watkins et al., 1994, 1995; DeLeo et al., 1997) and the discovery that the microglial reaction in the spinal cord and the development of neuropathic pain timely coincide (Colburn et al., 1997, 1999; Coyle, 1998) have raised the assumption that microglia are involved in neuropathic pain development (Watkins et al., 2001). It is clear today that inhibition of various microglia-specific receptors or effector molecules prevents the development of neuropathic pain (Jin et al., 2003; Schäfers et al., 2003; Tsuda et al., 2003; Terayama et al., 2008; Clark et al., 2009, 2010). Taken together, it is widely accepted that microglia function is crucial for the initiation of neuropathic pain (see for review: Ji et al., 2006; McMahon and Malcangio, 2009; Svensson and Brodin, 2010; Trang et al., 2012; Clark et al., 2013; Tsuda et al., 2013). However, while much has been revealed about the function of numerous microglia factors and receptors like P2X4, P2X7, TLR2, CX3CR1, BDNF and CatS (see fore excellent and recent reviews: Ji et al., 2006; McMahon and Malcangio, 2009; Svensson and Brodin, 2010; Trang et al., 2012; Clark et al., 2013; Tsuda et al., 2013) comparably little is yet know about the mechanisms that initiate the microglia response after peripheral nerve injury. From a therapeutically point of view, however, it would be of crucial interest to identify the signals that turn on the microglia response after peripheral nerve injury.

CHEMOKINES: EFFECTIVE SIGNALING MOLECULES IN THE BRAIN

The CNS is spatially highly organized. In general neuron-neuron communication in the CNS is based on the regulated release of various signaling molecules, like neurotransmitters, neuropeptides, neurohormones and neurotrophins. With few exceptions, the release of these signaling molecules occurs at specific sites, for example synapses between neurons. This specific release requires a targeted intracellular transport of signaling molecules to these sites. Accordingly, neurons have various systems for the sorting, transportation and release of their numerous signaling

molecules. Neurotransmitters are generally found in small, so-called synaptic vesicles, which undergo recycling and are loaded with neurotransmitters at the synapses. All protein or peptide signaling molecules are delivered to the membrane in either the constitutive or the regulated release pathway. This protein cargo is synthesized in the endoplasmatic reticulum (ER) and sorted in the trans-golgi-network (TGN) of the neurons. The vesicles of the regulated release pathway belong to the large dense core vesicles (LDV), with which neurons are able to sort, transport and release protein-signaling molecules like neurotrophins or neuropeptides at distinct sub-cellular sites (see for review: van Vliet et al., 2003; Salio et al., 2006; Gottmann et al., 2009; Zhang et al., 2010). Synapses between neurons are no longer considered the only communication points in the CNS since there is accumulating evidence for extrasynaptic release of signaling molecules and since there is considerable communication ongoing also between neurons and surrounding glia cells (Biber et al., 2007; Araque and Navarrete, 2010; Faissner et al., 2010; Giaume et al., 2010). Thus the concept of intracellular communication in the CNS has substantially broadened and therefore it is not surprising that new families of molecules are discussed at the moment to be messengers in the brain.

Chemokines are small proteins (10–20 kDa) and originally known from the peripheral immune system, where they orchestrate various aspects of immunity. Originally chemokines were described as chemotaxis-inducing cytokines; however, today it is clear that chemokines control numerous aspects of immune function making them important signaling molecules in health and disease (Borroni et al., 2010; Sharma, 2010). The first reports on chemokine expression in the brain focused on glia cells and their potential role in neuroimmunology (Biber et al., 2002). Apart from their expression in glia cells, at least five different chemokines (CCL2, CCL21, CXCL10, CXCL12 and CX3CL1) have been described in neurons in the last few years, predominately under conditions of neuronal stress or injury (de Haas et al., 2007; Biber et al., 2008; Miller et al., 2008). Since these chemokines have electrophysiological effects in neurons (Oh et al., 2002; Callewaere et al., 2006; Guyon et al., 2009; Miller et al., 2009) and control glia cell function in brain pathology (Cardona et al., 2008; Ransohoff, 2009), an important function of these neuronal chemokines in conveying signals from injured neurons has been suggested (de Haas et al., 2007; Ransohoff, 2009). The role of chemokines as microglia instruction signals has gained particular interest in the field of neuropathic pain, where at least three different neuronal chemokines (CX3XL1, CCL2 and CCL21) are playing different roles. Since the contribution of CX3CL1/CX3CR1 signaling in neuropathic pain is covered by Clark and Malcangio in this special research topic in Frontiers in Cellular Neuroscience (Clark and Malcangio, 2014), we here will focus on CCL2 and CCL21.

NEURONAL CCL2 AND CCL21 AND THEIR POTENTIAL ROLE IN NEUROPATHIC PAIN

The chemokines CCL2 and CCL21 have both been described to be up-regulated in injured DRG neurons (Zhang et al., 2007; Jung et al., 2009; Miller et al., 2009; Biber et al., 2011) and their role as neuron-microglia signaling factors involved in development of neuropathic pain has been proposed (Zhang et al., 2007; Jung et al., 2009; Miller et al., 2009; Biber et al., 2011). Both CCL2 and CCL21 are induced in the cell bodies of DRG neurons that are located outside of the spinal cord. There would be thus two prerequisites for effective microglia activation by neuronal chemokines in the spinal cord: first adequate transport of these chemokines from the DRG into the spinal cord is required and second spinal microglia should express of the corresponding receptors for CCL2 and CCL21.

SORTING AND TRANSPORT OF NEURONAL CCL21 AND CCL2

The first evidence that CCL21 is specifically expressed in endangered neurons and may act as a signal from damaged neurons to microglia was published more than a decade ago (Biber et al., 2001). In subsequent studies in mice with disturbed CCL21 signaling inhibited microglia responses at the projection site of injured neurons were found and it was speculated that CCL21 is transported to axon endings (Rappert et al., 2004; de Jong et al., 2005). Corroborating this assumption it was observed that neuronal CCL21 is located in vesicles in neuronal cell bodies, axons and pre-synaptic terminals (de Jong et al., 2005). Subsequently CCL21-containing vesicles were identified as LDVs and their preferential transport towards the axon ends was shown (de Jong et al., 2008). These data were recently confirmed in dorsal root ganglion cells, in which CCL21 expression is induced by mechanical injury with subsequent transport of CCL21 through the dorsal root into the primary afferents in the spinal cord (Biber et al., 2011).

Similarly there is solid evidence from various models of neuropathic pain that CCL2 is strongly upregulated in DRG neurons (Tanaka et al., 2004; White et al., 2005; Zhang and De Koninck, 2006; Yang et al., 2007; Jung et al., 2008, 2009; Bhangoo et al., 2009; Jeon et al., 2009; Thacker et al., 2009; Van Steenwinckel et al., 2011). There is however, conflicting evidence about the transport of CCL2 from the DRG into the dorsal horn of the spinal cord. Whereas immunohistochemical findings suggested the transport of CCL2 from the DRG into the spinal cord (Zhang and De Koninck, 2006; Thacker et al., 2009; Van Steenwinckel et al., 2011), a report on CCL2-mRFP1 expressing transgenic mice showed that CCL2 expression was restricted to the lesioned DRG (Jung et al., 2009). Since different lesion models of the spinal nerve were used in these studies the question whether or not CCL2 is transported from the DRG to the spinal cord might depend on the lesion model.

The transport of CCL2, however, would require that CCL2 (like CCL21) is sorted into vesicles that allow such transport. Indeed, there also is evidence that CCL2 is expressed in neuronal vesicles (Jung et al., 2009) and a recent report using electron microscopy described CCL2 expression in small clear vesicles and LDV (Van Steenwinckel et al., 2011) suggesting that like CCL21 also CCL2 is sorted into vesicles of the regulated release pathway which would allow its directed transport and release. However, the mechanism of how neuronal chemokines are being sorted into LDV is a yet not explored question.

The classic cargo of LDV like neurohormones, neuropeptides and neurotrophins are all synthesized in a pre-pro-form and sorted in the TGN (see for review: van Vliet et al., 2003; Salio

et al., 2006; Gottmann et al., 2009; Zhang et al., 2010). The "pre" of the pre-pro-form indicates the N-terminal signal peptide which is cleaved to allow the entry of the protein into the ER (van Vliet et al., 2003). Such N-terminal signal was also described for CCL21 and its deletion resulted in cytoplasmic expression of the chemokine showing that the entry into the ER is essential for the sorting of CCL21 (de Jong et al., 2008). Interestingly, bioinformatically methods using the online software SignalP3.0[1] would propose such N-terminal signal also for CCL2, which would be cleaved off between position 23 and 24. Whether or not the deletion of this proposed N-terminal signal would also result in cytoplasmic expression of CCL2 is currently not known. However, the entry into the ER only is the first step of the sorting procedure and also is required for cargo that is sorted into the constitutive release pathway (see for review: van Vliet et al., 2003; Salio et al., 2006; Gottmann et al., 2009; Zhang et al., 2010). For the further sorting of cargo of the regulated release pathway into LDVs various proteases are involved and there is convincing evidence that the processing of the pro-form is required for the differential sorting of the cargo. Accordingly, various molecular sorting signals in the pro-form of LDV cargo have been identified (see for review: van Vliet et al., 2003; Salio et al., 2006; Gottmann et al., 2009; Zhang et al., 2010).

In contrast to classical LDV cargo, neuronal chemokines are not synthesized in a pre-pro-form, but in a pre-form, meaning that they only have the N-terminal signal peptide allowing them to enter the ER. Therefore, it is currently not understood how exactly CCL21 and potentially CCL2 in neurons are subjected to specific sorting into LDVs. However, the fact that both CCL21 and most likely CCL2 are sorted into LDVs the possibility arises the possibility that both chemokines are transported to different locations in neurons.

Taken together, various lines of evidence show that nerve injury causes the expression of the chemokines CCL2 and CCL21 in peripheral neurons. After injury, their rapid expression first is detected in the cell bodies of the neurons lying peripherally in the DRG, after which both chemokines are most likely transported through the dorsal root into the primary afferents in the spinal cord. Thus both chemokines fulfil the first requirement of being a signal that conveys the message of nerve damage from the periphery into the spinal cord.

It is interesting to note here that CCL21 has yet never been detected in healthy neurons, glia cells or other non-neuronal cells in the brain such as endothelial cells. Thus, CCL21 in the CNS is exclusively expressed in injured neurons and thus is one the few inflammatory mediators in the CNS with such exclusive cell specificity indicating a special role of this chemokine for the communication between injured neurons and their surroundings. In contrast, next to its neuronal expression, CCL2 in the brain has been additionally described in glia cells (astrocytes, microglia) (Biber et al., 2002). Furthermore, in peripheral nerve injury and development of neuropathic pain expression of CCL2 has been described in other cells than the injured DRG neurons, indicating that being a potential message to microglia most likely is not the only function of CCL2 after peripheral nerve injury (see below).

[1]http://www.cbs.dtu.dk/services/SignalP/

CCR2: A CHEMOKINE RECEPTOR IN MICROGLIA?

Since microglia are of myeloid origin and share many properties with peripheral monocytes/macrophages it was expected that microglia express the receptor for CCL2, formerly called monocyte chemoattractant protein-1 (MCP-1). There are thus various reports in which CCR2 expressing cells are suggested to be microglia (Abbadie et al., 2003; Zhang et al., 2007; Fernández-López et al., 2012) or described as microglia/macrophages (Yao and Tsirka, 2012) or referred to as amoeboid microglia cells (Deng et al., 2009). Often CCR2 is discussed to be an important receptor for the recruitment of microglia to injured brain areas (El Khoury et al., 2007; Zhang et al., 2007; Deng et al., 2009; Raber et al., 2013) and in this respect CCR2 has been described as receptor in spinal cord microglia that enables these cells to respond to peripheral nerve injury (Abbadie et al., 2003; Zhang et al., 2007).

On the other hand there is convincing evidence that microglia do *not* express CCR2. Various recent mRNA expression studies in acutely isolated microglia from the adult mouse brain did not detect CCR2 mRNA expression in these cells (Olah et al., 2012; Beutner et al., 2013; Hickman et al., 2013; Butovsky et al., 2014) nor was CCR2 mRNA expression earlier found in cultured microglia (Zuurman et al., 2003). Two different studies using transgenic mouse models in which CCR2-expressing cells were fluorescently labelled failed to detect the corresponding fluorescent signal in microglia in the healthy brain and in various disease models such as experimental autoimmune encephalomyelitis (EAE), LPS-injection and sciatic nerve demyelination (Jung et al., 2009; Mizutani et al., 2012). Finally there are various bone-marrow transplantation studies and experiments with parabiotic mice that show CCR2 expression solely in peripheral monocytes/macrophages that have invaded the diseased central nervous system (Mildner et al., 2007; Schilling et al., 2009a,b; Prinz and Mildner, 2011; Mizutani et al., 2012).

How is this controversy around CCR2 expression in microglia explained? With respect to their origin it is clear now that microglia are derived from primitive c-kit+ erythromyeloid yolk sac precursor cells that appear as early as embryonic day 8 in the mouse (Ginhoux et al., 2010; Kierdorf et al., 2013). Importantly, only these cells invade the developing nervous tissue and mature into microglia. Microglia never exchange with cells that stem from fetal liver- or bone-marrow haematopoiesis, making microglia a myeloid cell population in the adult that is exclusively derived from primitive haematopoiesis (Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013). Microglia therefore are a specialized and local cell population, that most likely display self-renewing capacities without exchange with peripheral cells under physiological conditions (Ajami et al., 2007; Ginhoux et al., 2013). Since CCR2+/Lys6C high inflammatory monocytes, the cells that may enter the diseased brain, are derived from definitive haematopoiesis they are of different origin as microglia, yet it is extremely difficult to distinguish both populations in the diseased brain (see for recent review: Ginhoux et al., 2013; Neumann and Wekerle, 2013; Biber et al., 2014). Since it was shown that peripheral nerve injury led to a rapid (within 24 h) and transient (up to 7 days) opening of the blood-spinal cord barrier (Beggs et al., 2010) and that CCR2-postive peripheral cells enter the spinal cord in response to peripheral nerve injury (Zhang

et al., 2007), the controversy about CCR2 expression in spinal cord microglia could potentially be due to CCR2+ inflammatory monocytes that have entered the spinal cord where they have been mistaken for endogenous microglia.

The lack of CCR2 in microglia would not support a role for neuronal CCL2 as microglia signal, however, the importance of CCL2 and its receptor CCR2 for the development of nerve-injury induced neuropathic pain is undisputed. There is an overwhelming body of literature that interfering with the CCL2-CCR2 system (antagonists, knockouts, inhibitor studies) reduces or prevents the development of neuropathic pain (see for recent reviews: Gao and Ji, 2010; Clark et al., 2013). It is obvious that the role of CCL2-CCR2 in this pathological pain state is mnifold and likely acts on various levels. Given the known role of CCL2 as an attracting factor for peripheral myeloid cells in the CNS it is most likely that CCL2 also in the spinal cord is important for the infiltration with monocytes/macrophages (Zhang et al., 2007). However, CCR2 is not only expressed in peripheral myeloid cells but also in DRG neurons and potentially in second order neurons in lamina II of the spinal cord (Gao et al., 2009; Jung et al., 2009). In these neurons several pro-nociceptive electrophysiological effects of CCL2 like enhancement of enhance glutamate receptor function or reduction of GABAergic signaling (Gosselin et al., 2005; Gao et al., 2009; Gao and Ji, 2010; Clark et al., 2013). Thus CCL2 in the DRG may act as autocrine signal (neuron-neuron signal) and paracrine in the spinal cord where neuronally released CCL2 may stimulate second order neurons in the pain cascade. The primary afferents of the DRG neurons are, however not the only cellular source of CCL2, as also spinal cord astrocytes express CCL2 under conditions of neuropathic pain (Gao and Ji, 2010; Clark et al., 2013). Thus interfering with CCL2 signaling may inhibit neuropathic pain development at various levels. Since microglia responses and neuropathic pain development are closely connected to each other, it may very well be that an inhibition of the pain cascade (by CCL2 antagonists for example) also inhibits the pain-related reaction of microglia. Such findings, however, are no formal proof of a direct effect of CCL2 in microglia.

CCL21 RECEPTORS IN MICROGLIA

Using CCL21-deficient mice (plt mutation) an important role of this neuronal chemokine in the development of neuropathic pain was demonstrated. Without neuronal CCL21 expression, animals did not develop signs of tactile allodynia in response to spinal nerve injury (Biber et al., 2011). This lack of neuropathic pain was due to a failure in microglia to up-regulate P2X4 expression after spinal nerve injury (Biber et al., 2011). In cultured microglia P2X4 mRNA and protein was induced by CCL21 stimulation showing that this chemokine is the responsible neuronal trigger for P2X4 up-regulation in microglia and the development of neuropathic pain (Biber et al., 2011), raising the question which microglia receptor is responsible here.

There are two known receptors for CCL21 in mice: CCR7 and CXCR3 (Biber et al., 2006). The main receptor for CCL21 is CCR7, which is not found in microglia under basal conditions, but it can be induced *in vitro* and *in vivo* (Biber et al., 2001, 2002; Rappert et al., 2002; Dijkstra et al., 2006). In contrast, CXCR3 is constitutively expressed in cultured microglia and in acutely isolated microglia (Biber et al., 2001, 2002; Rappert et al., 2002; de Haas et al., 2008). Thus cultured non-challenged microglia from CXCR3-deficient animals are not responsive to CCL21 stimulation (Rappert et al., 2002) but gain reactivity to CCL21 after immunological challenges (Dijkstra et al., 2006). Furthermore, CXCR3-deficient animals display markedly reduced microglia activation after neuronal injury in the entorhinal cortex lesion model (Rappert et al., 2004), indicating a prominent role of CXCR3 in microglia for the detection of neuronal damage in the nervous system. In order to understand which CCL21 receptor is involved in the development of neuropathic pain, CCR7-/- and CXCR3-/- animals were subjected to peripheral nerve damage. CCR7-deficient animals displayed a somewhat milder disease course, especially during the first days after spinal nerve injury (Biber et al., 2011). This delay in allodynia development might point to an induction of CCR7 expression in activated dorsal horn microglia, similar to what was found in a mouse model of multiple sclerosis (Dijkstra et al., 2006). However, in agreement with earlier studies we were not able to detect any CCR7 mRNA in the control spinal cord, neither was CCR7 mRNA induced by the nerve lesion. Given this lack of CCR7 in spinal cord tissue, the slightly milder disease development after spinal nerve injury in CCR7-deficient animals is most likely due to a yet not understood effect in the periphery. Surprisingly, the development of neuropathic pain was also not affected in CXCR3-deficient animals (Biber et al., 2011). Thus neither the deficiency of CCR7 or CXCR3 had a profound impact on the development of neuropathic pain, in contrast to the striking phenotype in the absence of their ligand CCL21.

The fact that only CCL21, but not the specific CXCR3 ligand CXCL10 or the specific CCR7 ligand CCL19 were able to induce P2X4 mRNA expression in cultured mouse microglia might point to another CCL21 receptor in these cells. Indeed, we have recently provided functional evidence for a third, yet not identified, CCL21 receptor in mouse glia cells (van Weering et al., 2010), indicating that the question of CCL21 receptors in glia cells is more complex than originally anticipated. Taken together, the responsible receptor for the CCL21-dependent development of neuropathic pain after spinal nerve injury remains to be established.

CONCLUSIONS

Despite the similar expression pattern in response to peripheral nerve injury there are clear differences in function of neuronal CCL2 and CCL21 in the development of neuropathic pain (**Figure 1**). CCL2 in the injured DRG may act as local autocrine signal (neuron-neuron signal) and paracrine in the spinal cord where neuronally released CCL2 may stimulate second order neurons in the pain cascade and/or attract CCR2-expressing peripheral monocytes/macrophages. Neuronal CCL21 contributes to neuron-microglia signaling and is the crucial trigger to up-regulate P2X4 receptors in spinal cord microglia, a vital step in the cascade that leads to neuropathic pain. Thus both neuronal chemokines play important roles in neuropathic pain development are potential drug targets to prevent the formation of neuropathic pain in response to peripheral nerve injury.

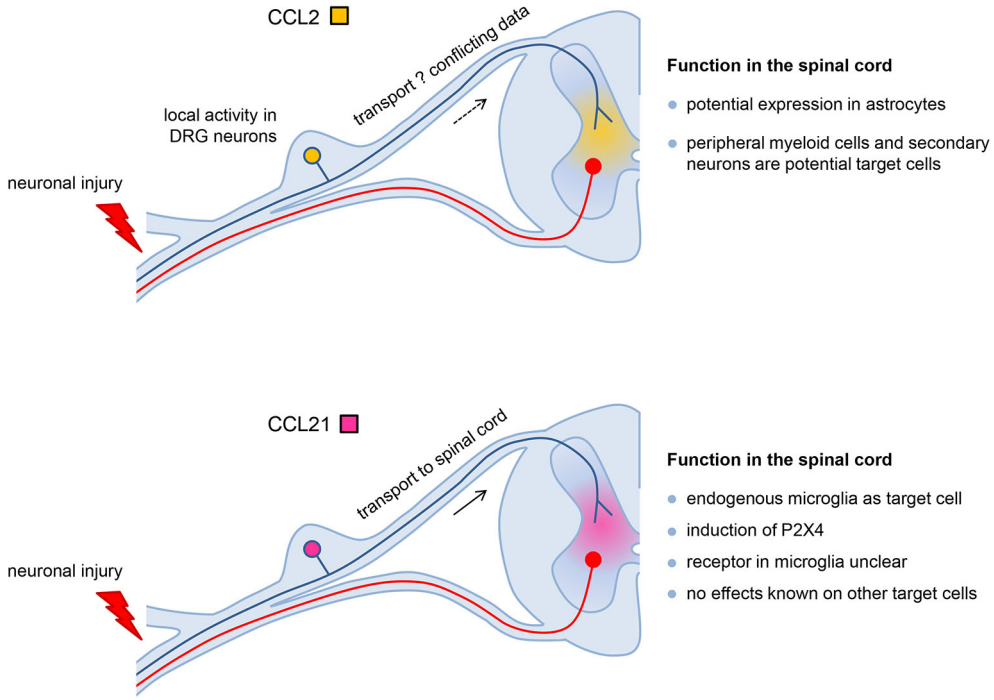

FIGURE 1 | The different roles of CCL2 and CCL21 in the development of neuropathic pain. Both chemokines are induced in DRG neurons in response to nerve injury. CCL2 in the injured DRG may act as local autocrine signal (neuron-neuron signal) and potentially paracrine in the spinal cord where neuronally released CCL2 may stimulate second order neurons in the pain cascade and/or attract CCR2-expressing peripheral monocytes/macrophages. Since there are conflicting data about the transport of CCL2 from the DRG into the spinal cord, alternatively CCL2 from astrocytes might also activate these target cells. Neuronal CCL21 is transported from the DRG into the spinal cord and contributes to neuron-microglia signaling. CCL21 is the crucial trigger to up-regulate P2X4 receptors in spinal cord microglia which is a vital step in the cascade that leads to neuropathic pain. Although the receptor for CCL21 in spinal cord microglia is an unsolved issue, this chemokine most likely acts as neuron-microglia signal only, since effects of CCL21 in other cells of the spinal cord have yet not been described.

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Fractalkine/CX3CR1 signaling during neuropathic pain

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Chronic pain represents a major problem in clinical medicine. Whilst the acute pain that is associated with tissue injury is a protective signal that serves to maintain homeostasis, chronic pain is a debilitating condition that persists long after the inciting stimulus subsides. Chronic neuropathic pain that develops following damage or disease of the nervous system is partially treated by current therapies, leaving scope for new therapies to improve treatment outcome. Peripheral nerve damage is associated with alterations to the sensory neuroaxis that promote maladaptive augmentation of nociceptive transmission. Thus, neuropathic pain patients exhibit exaggerated responses to noxious stimuli, as well as pain caused by stimuli which are normally non-painful. Increased nociceptive input from the periphery triggers physiological plasticity and long lasting transcriptional and post-translational changes in the CNS defined as central sensitization. Nerve injury induces gliosis which contributes to central sensitization and results in enhanced communication between neurons and microglial cells within the dorsal horn. Thus, identification of mechanisms regulating neuro-immune interactions that occur during neuropathic pain may provide future therapeutic targets. Specifically, chemokines and their receptors play a pivotal role in mediating neuro-immune communication which leads to increased nociception. In particular, the chemokine Fractalkine (FKN) and the CX3CR1 receptor have come to light as a key signaling pair during neuropathic pain states.

Keywords: microglia, proteases, pain, chronic pain, chemokines

INTRODUCTION

Acute pain can be regarded as a homeostatic and adaptive process by which the organism becomes aware of harmful stimuli, thus guarding against actual or potential tissue injury. As such, the physiological transduction and transmission of noxious stimuli is a vital protective mechanism (nociceptive pain), allowing withdrawal from potentially damaging environmental factors. Nociceptive pain persists only for the duration of the stimulus or tissue damage. The fundamental importance of pain as a homeostatic mechanism becomes apparent in the case of individuals who have a complete lack of nociception; rare hereditary mutations resulting in congenital insensitivity to pain lead affected individuals to inadvertently inflict injury upon themselves throughout life (Cox et al., 2006).

Under some circumstances pain can outlast its physiological role, developing into chronic pain; a debilitating condition lasting longer than 3 months from the noxious stimulus, during which the pain is out of proportion to the initial inciting injury. Chronic neuropathic pain results from damage to, or dysfunction of, the somatosensory system and is maladaptive in that the pain neither protects the organism nor supports tissue repair. Neuropathic pain is commonly associated with direct trauma (stretch or crush) to a peripheral nerve. In addition, disease states including diabetes mellitus and viral infections may result in neuropathic pain symptoms. Furthermore, pharmacological agents such as anti-retroviral drugs and chemotherapy agents may also result in the development of painful neuropathy following dysfunction of sensory nerves. Neuropathic pain is a complex pain syndrome consisting of multiple symptoms. These include sensory loss, abnormal sensation, spontaneous pain, and alterations in responses to stimulus-evoked pain (hyperalgesia and allodynia) (Jensen et al., 2001; Baron, 2006). Neuropathic pain is a significant clinical problem, for which current treatments are inadequate. This is due in large part to the fact that the mechanisms underlying neuropathic pain syndromes are insufficiently understood.

Convincing pre-clinical evidence suggests that following peripheral nerve injury neuro-immune interactions play pivotal roles in the generation and maintenance of nociceptive hypersensitivity. Cells of the immune system interact with the sensory system at various locations. In the peripheral nerve the infiltration of immune cells (which release both pro-nociceptive and anti-nociceptive mediators) is critical for the early initiation phase of neuropathic pain in rodent models (Austin and Moalem-Taylor, 2010; Stein and Machelska, 2011). In the dorsal horn of the spinal cord disruption of homeostasis and exaggerated primary afferent input causes microglia to transition from surveillance states into pain-related enhanced response states, thus modifying the nature of neuron-microglia communication and promoting a maladaptive augmentation of nociceptive transmission that underlies the chronicity of neuropathic pain.

Neuron-microglia communications in the dorsal horn occur through activation of defined pathways. In particular, two critical neuron-microglia signaling systems initiated by purinergic

receptors contribute to nerve injury induced hypersensitivity. A microglia-driven pathway whereby *de novo* P2X4 receptor expression and activation leads to release of Brain-Derived Neurotrophic Factor (BDNF; Ulmann et al., 2008; Trang et al., 2009) is critical during the initiation phase of neuropathic pain (shortly after nerve injury) (Tsuda et al., 2003). BDNF activation of the TrkB receptor down-regulates the expression of the neuronal potassium/chloride co-transporter KCC2 (Coull et al., 2005). The consequential impairment of chloride homeostasis in the superficial laminae of the dorsal horn results in reduced inhibition following $GABA_A$ receptor activation (Coull et al., 2005), and therefore a more excitatory environment. The therapeutic exploitability of this P2X4/BDNF/KCC2 pathway is highlighted by the recent identification of chloride extrusion enhancer compounds that exert significant anti-nociceptive effects in neuropathic rats (Gagnon et al., 2013).

We have identified a second neuron-microglia signaling pathway that is critically involved in the maintenance phase of neuropathic pain. This second microglia-driven pathway is initiated by activation of the low affinity P2X7 receptor, resulting in release of the lysosomal protease Cathepsin S (CatS; Clark et al., 2010). This protease maintains activity at neutral pH and can liberate the chemokine domain of the neuronal chemokine Fractalkine (FKN), which feeds back onto microglia through the engagement of the CX3CR1 receptor (Clark et al., 2007, 2009). Here we review the contribution of spinal FKN/CX3CR1 signaling to neuroimmune interactions during neuropathic pain.

THE FKN/CX3CR1 SIGNALING PAIR

Chemokines generally have a promiscuous relationship with their G-protein coupled receptors, with one chemokine binding to several different receptors and one receptor binding a range of ligands. However, the chemokine system is not functionally redundant (Schall and Proudfoot, 2011). One chemokine interaction, between FKN (CX3CL1) and its receptor CX3CR1, is a monogamous relationship. In addition, FKN is structurally unique amongst the family of chemokines; it is the only member of the CX3C family of chemokines and was first described as a potent attractant of immune cells (Bazan et al., 1997; Pan et al., 1997). The protein can exist in two forms, each of which mediates distinct biological actions: a membrane tethered protein and soluble forms containing the chemokine domain (Bazan et al., 1997).

FKN is expressed in both the periphery and the CNS. Pan et al. originally described FKN gene expression to be most abundant in the brain and heart, but absent from peripheral blood leukocytes (Pan et al., 1997). Endothelial and epithelial cells are the predominant FKN-expressing cells in the periphery. Indeed, FKN has been localized to endothelial cells of the skin (Papadopoulos et al., 1999, 2000), heart (Harrison et al., 1999), and lung (Foussat et al., 2000), and to intestinal epithelial and endothelial cells (Muehlhoefer et al., 2000). This constitutive expression of FKN is regulated by inflammatory stimuli; it is enhanced following exposure of these cells to Lipopolysaccharide (LPS; Pan et al., 1997), pro-inflammatory cytokines (Bazan et al., 1997; Muehlhoefer et al., 2000), and during inflammatory conditions such as Crohn's disease (Muehlhoefer et al., 2000).

Neurons are the principle FKN expressing cells of the CNS, with endothelial cells in the brain showing little or no expression (Harrison et al., 1998; Nishiyori et al., 1998; Maciejewski et al., 1999; Hughes et al., 2002; Tarozzo et al., 2002, 2003). Likewise in the spinal cord FKN expression is restricted to neurons (Verge et al., 2004; Lindia et al., 2005; Clark et al., 2009; Yang et al., 2012). FKN expression has also been observed in the cell bodies of peripheral sensory neurons in the dorsal root ganglia (DRG; Verge et al., 2004), and in the central terminals of these neurons in the spinal dorsal horn in some studies (Verge et al., 2004; Yang et al., 2012), but not in others (Lindia et al., 2005; Clark et al., 2009). The expression profile of FKN has been confirmed by the recent development of a FKN reporter mouse (Kim et al., 2011). Peripherally, the expression of FKN in these mice is completely restricted to non-hematopoietic cells, with FKN-mCherry found in lung and intestinal epithelial cells and in kidney endothelial cells (Kim et al., 2011). Centrally, the steady-state neuronal location of FKN in some brain areas (hippocampus, striatum and cortical layer II) and spinal cord was also confirmed. However, FKN-mCherry expression was absent from the brainstem, midbrain, and cerebellum. FKN-mCherry was also not found in DRG cells (Kim et al., 2011), somehow questioning sensory neurons as a source of FKN outside the CNS under homeostatic conditions.

The shedding of membrane bound FKN into soluble forms represents a key regulatory mechanism for FKN signaling. The liberation of soluble FKN (sFKN) from endothelial and epithelial cells occurs both constitutively and in an inducible manner. In the context of vascular immune function, endothelial membrane bound FKN serves as an adhesion molecule, promoting the firm adhesion of leukocytes without the activation of integrins (Fong et al., 1998), whilst sFKN is a potent chemoattractant for monocytes, NK cells, T cells and B cells (Imai et al., 1997; Corcione et al., 2009). FKN/CX3CR1 interactions are also vital for many homeostatic processes, including the survival of $CX3CR1^{high}$ blood monocytes (Landsman et al., 2009), wound healing (Ishida et al., 2008) and trans-endothelial migration for immune surveillance (Auffray et al., 2007). Constitutive shedding of membrane bound FKN is principally dependent on the metalloprotease ADAM-10 (a disintegrin and metalloprotease domain-10) (Hundhausen et al., 2003, 2007). Following stimulation of FKN-expressing cells with phorbol esters (e.g., Phorbol 12-myristate 13-acetate) shedding of mature FKN (~100 kDa) into soluble FKN (~80 kDa) is markedly enhanced; this inducible shedding is largely ADAM-17 (also known as TACE, tumor necrosis factor-α converting enzyme) dependent (Garton et al., 2001; Tsou et al., 2001). However, not all shedding of FKN observed can be accounted for by cleavage of ADAM-10 and ADAM-17, as following metalloproteinase inhibition some formation of sFKN is still observed (Hundhausen et al., 2003). Recent evidence indicates that the cysteine protease CatS expressed by vascular smooth cells also generates sFKN, although of a smaller size (~50 kDa) (Fonović et al., 2013) than the sFKN liberated by the ADAMs. Indeed, in the spinal cord during chronic pain sFKN is liberated following cleavage of neuronal membrane bound FKN by CatS released by microglia (Clark et al., 2007, 2009). The possibility that ADAM-17 and/or ADAM-10 contributes to sFKN

shedding in the spinal cord has not been evaluated, however FKN expression is absent from CNS endothelium (Harrison et al., 1998; Nishiyori et al., 1998; Maciejewski et al., 1999; Hughes et al., 2002; Tarozzo et al., 2002, 2003), therefore ADAM mediated cleavage of FKN in the CNS seems unlikely. Interestingly, different proteases may cleave FKN at diverse locations and it is likely that sFKN exists in several forms. ADAM-10 and ADAM-17 cleave FKN at different sites close to the plasma membrane (Bazan et al., 1997; Garton et al., 2001; Tsou et al., 2001), whilst the exact cleavage site of CatS has not yet been determined.

The CX3CR1 receptor was identified in humans (Imai et al., 1997; Combadiere et al., 1998) and rat (Harrison et al., 1994) in the 1990's. Like all of the chemokine receptors, CX3CR1 is seven-transmembrane domained G-protein coupled receptor. CX3CR1 expression is abundant in both peripheral blood leukocytes and microglia in the CNS. The development of a transgenic mouse by Jung et al. in which the CX3CR1 gene was mutated to contain a green fluorescent protein (GFP) reporter gene (Jung et al., 2000), has allowed the pattern of CX3CR1 expression in the mouse to be analyzed in depth. Murine blood contains populations of monocytes ($CD11b^{+}$ $Gr1^{low}$) and Natural Killer cells that express CX3CR1. On the other hand, murine B-lymphocytes and T-lymphocytes (both resting and active), eosinophils and neutrophils are CX3CR1 negative. Expression of CX3CR1 is also found on both myeloid and lymphoid dendritic cells and populations of cutaneous Langerhans cells (Jung et al., 2000). It should be noted that the expression of CX3CR1 in human blood differs from that in the mouse, with expression observed in populations of human T-lymphocytes (Raport et al., 1995; Foussat et al., 2000). In the CNS, CX3CR1 is exclusively expressed by microglia. In both the mouse and the rat microglia in the brain express CX3CR1, with expression completely absent from astrocytes, oligodendrocytes and neurons (Harrison et al., 1998; Nishiyori et al., 1998; Jung et al., 2000). Likewise in the spinal cord CX3CR1 is exclusively expressed by microglial cells (Verge et al., 2004; Lindia et al., 2005; Zhuang et al., 2007; Yang et al., 2012; Clark et al., 2013). Controversial *in vitro* evidence for neuronal CX3CR1 expression in cultured hippocampal neurons (Meucci et al., 2000; Limatola et al., 2005), has not been confirmed *in vivo* using the CX3CR1-GFP reporter mouse (Jung et al., 2000), suggesting that such expression may be a phenomenon of the culture system. Critically the neuroprotective effects of FKN in hippocampal cultures originally attributed to a direct action on the hippocampal neurons themselves (Meucci et al., 2000), has been demonstrated to be mediated by microglial released mediators, and can be attributed to microglial contamination in the neuronal cultures (Lauro et al., 2008). Overall evidence indicates that in the CNS the FKN/CX3CR1 signaling pair are ideally located to mediate neuron-microglial communication, both during homeostatic and pathological processes.

In the brain FKN/CX3CR1 interactions are thought to play a homeostatic role in the regulation of microglia cell activity, contributing to the maintenance of a surveillance state in these cells. It has been demonstrated that FKN/CX3CR1 regulate hippocampal neurogenesis, synaptic pruning, synaptic plasticity, and are neuroprotective in a number of pathological conditions (Recently reviewed in Sheridan and Murphy, 2013). The role of FKN/CX3CR1 interactions in spinal homeostatic mechanisms remains to be determined. However, it has become evident that aberrant FKN/CX3CR1 signaling can contribute significantly to the pathogenesis of a number of chronic diseases (Nishimura et al., 2009; Jones et al., 2010; Clark et al., 2011; Liu and Jiang, 2011), perhaps unsurprising given the role of this pair in immune and inflammatory processes. Among these conditions, there is now extensive evidence to support a role for FKN/CX3CR1 signaling in the chronicity of pain.

SPINAL FKN/CX3CR1 AND NEURON-MICROGLIA COMMUNICATION DURING NEUROPATHIC PAIN

The first synapse in the nociceptive pathway, between the central terminals of primary afferent fibers and dorsal horn neurons in the spinal cord, is a key site at which modulation of nociceptive transmission can occur. Neuropathic pain is commonly modeled in rodents using surgical injury to a peripheral nerve, usually the sciatic nerve or a branch thereof, which induces robust and reproducible pain behaviors in the effected hind-paw. It is now well established that damage to a peripheral nerve causes disruption of homeostasis; as a result microglia (and astrocytes) in the vicinity of injured primary afferent terminals in the dorsal horn transition into pain-related enhanced response states (McMahon and Malcangio, 2009). Thus augmentation of neuron-microglia communication critically contributes to amplification of nociceptive transmission which occurs during neuropathic pain. In the dorsal horn, neuronal FKN and microglial CX3CR1 are ideally located to mediate neuron-microglia communication.

FKN in its soluble form is pro-nociceptive; intrathecal administration of the FKN chemokine domain (Milligan et al., 2004, 2005; Clark et al., 2007; Zhuang et al., 2007; Clark and Malcangio, 2012), but not full length FKN (Clark and Malcangio, 2012), induces hypersensitivity to both thermal and mechanical stimuli, which is entirely mediated via CX3CR1 (Milligan et al., 2004, 2005; Clark et al., 2007; Staniland et al., 2010). FKN induces nociceptive behaviors following activation of CX3CR1 and intracellular phosphorylation of microglial p38 Mitogen-activated protein kinase (MAPK; Clark et al., 2007; Zhuang et al., 2007) which subsequently stimulates release of pro-inflammatory mediators including Interleukin-1β, Interleukin-6 and Nitric Oxide (Milligan et al., 2005).

Impairment of spinal FKN/CX3CR1 signaling represents a potential therapeutic avenue during chronic pain. Following injury to a peripheral nerve extensive upregulation of CX3CR1 occurs in spinal microglia (Verge et al., 2004; Lindia et al., 2005; Zhuang et al., 2007; Staniland et al., 2010), with FKN becoming *de novo* expressed in astrocytes in the spinal nerve transection model of peripheral nerve injury (Lindia et al., 2005), but not in other models (Verge et al., 2004; Zhuang et al., 2007; Staniland et al., 2010). Although levels of total FKN protein in the spinal cord remain unchanged following nerve injury (Verge et al., 2004; Lindia et al., 2005; Clark et al., 2009), sFKN levels in CSF are significantly elevated (Clark et al., 2009); thus there is enhanced availability of sFKN alongside enhanced CX3CR1 expression during neuropathic pain. In a number of models of

peripheral nerve injury intrathecal administration of FKN or CX3CR1 neutralizing antibodies is able to attenuate neuropathic pain behaviors (Milligan et al., 2004; Clark et al., 2007; Zhuang et al., 2007); this is due to a reduced pro-nociceptive activity state of spinal microglia, as demonstrated by reduced p38 MAPK phosphorylation (Zhuang et al., 2007). The same effect is true for the development of bone cancer pain; the development of pain in animals with experimental bone cancer occurs concurrently with microgliosis and an increase in the expression of microglial CX3CR1 and p-p38. The onset of this pain can be significantly delayed by the intrathecal administration of a CX3CR1 neutralizing antibody (Yin et al., 2010; Hu et al., 2012) despite a lack of efficacy in suppressing bone pathology (Yin et al., 2010). Whilst neutralizing antibodies and modified FKN proteins have been utilized for proof of concept preclinical studies, the first CX3CR1 antagonist to show anti-inflammatory activity at both mouse and human CX3CR1 was recently described (White et al., 2010; Karlström et al., 2013).

Critically, we demonstrated that CX3CR1 deficient mice show deficits in neuropathic pain; these mice do not develop mechanical allodynia, and have reduced hypersensitivity to thermal stimuli, following peripheral nerve injury, compared to wild-type mice (Staniland et al., 2010). The deficits in the development of neuropathic pain behaviors correlate with a reduction in microglial cell activity in these mice, as spinal microglial response is milder in knockout mice. Interestingly, extensive infiltration of macrophages occurs at the site of nerve injury; however no difference in the number of infiltration macrophages was

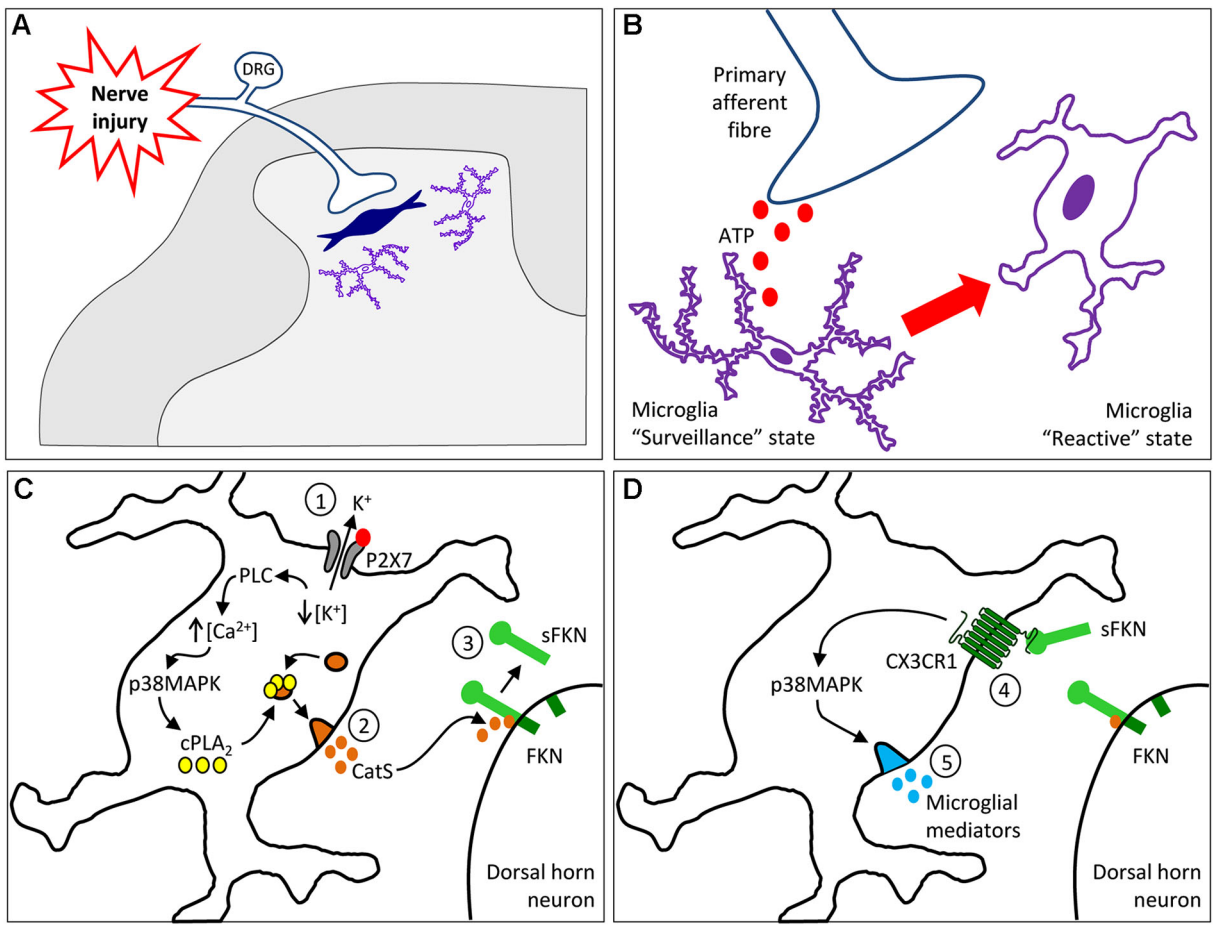

FIGURE 1 | Schematic illustrating the pro-nociceptive mechanism of CatS/FKN signaling in the spinal dorsal horn during neuropathic pain. (A–B) In the dorsal horn area innervated by damaged fibers (**Panel A**) microglia transform from a surveillance state into a reactive state following exposure to injury induced factors released by primary afferent terminals, including Adenosine tri-phosphate (ATP; **Panel B**). **(C)** High concentrations of extracellular ATP leads to P2X7 receptor activation on microglia (1), which ultimately leads to the release of CatS. A decrease in intracellular potassium concentration following efflux through the P2X7 receptor activates phospholipase C (PLC), resulting in an increase in intracellular calcium and phosphorylation of p38 MAPK. P38 phosphorylation then allows phospholipase A_2 (PLA_2) mediated translocation of CatS containing lysosomes to the cell membrane, whereby exocytosis releases CatS into the extracellular space (2). Extracellular CatS is then able to cleave membrane bound FKN from dorsal horn neurons, liberating soluble FKN (sFKN) (3). **(D)** sFKN feeds back onto the microglial cells via the CX3CR1 receptor (4) to further activate the p38 MAPK pathway and release inflammatory mediators, (5) that activate neurons and result in chronic pain. Abbreviations: DRG, dorsal root ganglia, $cPLA_2$, cytosolic PLA_2.

identified between genotypes (Staniland et al., 2010), suggesting that CX3CR1 expressing macrophages in the nerve contribute little to neuropathic pain in this model. In the spinal cord the pro-nociceptive actions of sFKN are mediated following its liberation by the lysosomal protease CatS (Recently reviewed in Clark and Malcangio, 2012). Following peripheral nerve injury CatS is upregulated in microglial cells in the area innervated by damaged primary afferent terminals (Clark et al., 2007). CatS is released from microglia in a P2X7 dependent manner (Clark et al., 2010), cleaving FKN located on the cell membrane of dorsal horn neurons to liberate the soluble chemokine domain of FKN, which then signals to microglia via CX3CR1 (Clark et al., 2007) (as summarized in **Figure 1**). Following peripheral nerve injury significant levels of sFKN can be detected in the CSF, along with enhanced CatS activity (Clark et al., 2009). FKN cleavage in the dorsal horn occurs under highly regulated conditions associated with increased nociception (Clark et al., 2009). In neuropathic spinal cord slices electrical stimulation of injured dorsal roots induces liberation of sFKN (Clark et al., 2009). The liberation of sFKN is only associated with conditions in which microglia are in an reactive state, for example following nerve injury or stimulation with LPS, and is completely dependent on CatS activity (Clark et al., 2009). Indeed, impairment of FKN signaling, either by neutralization of spinal FKN or by knockout of CX3CR1, is able to completely prevent the pro-nociceptive effects of intrathecal CatS (Clark et al., 2007).

The pro-nociceptive effects of the CatS/FKN/CX3CR1 signaling are critical for the maintenance phase of neuropathic pain. Both intrathecal (Clark et al., 2007) and systemic (Barclay et al., 2007; Irie et al., 2008; Zhang et al., 2014) delivery of CatS inhibitors reverse established pain behaviors following peripheral nerve injury to varying degrees. We have shown that CatS inhibitors are ineffective when given intrathecally during the initiation phase of neuropathic pain (at day 3 post-injury) (Clark et al., 2007) when expression levels are low both peripherally (Barclay et al., 2007) and in the spinal cord (Clark et al., 2007), but effectively reverse established pain behavior when delivered intrathecally at later timepoints when expression of CatS is high (Clark et al., 2007). Indeed, a recent study has confirmed our findings, demonstrating that when administered systemically an inhibitor of CatS reverses neuropathic pain behaviors commencing on day 5 post-injury, but is ineffective when delivered between day 0 and 4 (Zhang et al., 2014). In addition, CatS null mice develop pain behavior that is equivalent to wild-type mice immediately following nerve injury, only demonstrating a reduction in allodynia compared to wild-types from day 3 post-injury onwards (Zhang et al., 2014).

In summary, following peripheral nerve injury disruption of homeostasis leads to microglia-driven aberrant FKN/CX3CR1 signaling in the dorsal horn of the spinal cord which maintains maladaptive neuron-microglia signaling and critically contributes to the chronicity of neuropathic pain.

CONCLUSIONS

A greater understanding of the nature of neuron-microglia interactions during neuropathic pain states has led to the identification of new microglial therapeutic targets, including chemokine receptors such as CX3CR1 and the lysosomal protease CatS (Clark et al., 2011; Clark and Malcangio, 2012). Intracellular signaling pathways, most prominently p38 MAPK phosphorylation, mediate the release of pro-nociceptive mediators by spinal microglial cells comprising cytokines and proteases. Accordingly, the inhibition of microglial targets including CX3CR1, p38 MAPK and CatS can attenuate mechanical hypersensitivity in chronic pain models. Importantly, a CNS penetrant p38 MAPK inhibitor has demonstrated initial success in neuropathic pain patients (Anand et al., 2011) suggesting that impedance of microglial targets is a promising therapeutic avenue.

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Peroxisome proliferator-activated receptor agonists modulate neuropathic pain: a link to chemokines?

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Chronic pain presents a widespread and intractable medical problem. While numerous pharmaceuticals are used to treat chronic pain, drugs that are safe for extended use and highly effective at treating the most severe pain do not yet exist. Chronic pain resulting from nervous system injury (neuropathic pain) is common in conditions ranging from multiple sclerosis to HIV-1 infection to type II diabetes. Inflammation caused by neuropathy is believed to contribute to the generation and maintenance of neuropathic pain. Chemokines are key inflammatory mediators, several of which (MCP-1, RANTES, MIP-1α, fractalkine, SDF-1 among others) have been linked to chronic, neuropathic pain in both human conditions and animal models. The important roles chemokines play in inflammation and pain make them an attractive therapeutic target. Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors known for their roles in metabolism. Recent research has revealed that PPARs also play a role in inflammatory gene repression. PPAR agonists have wide-ranging effects including inhibition of chemokine expression and pain behavior reduction in animal models. Experimental evidence suggests a connection between the pain ameliorating effects of PPAR agonists and suppression of inflammatory gene expression, including chemokines. In early clinical research, one PPARα agonist, palmitoylethanolamide (PEA), shows promise in relieving chronic pain. If this link can be better established, PPAR agonists may represent a new drug therapy for neuropathic pain.

Keywords: neuropathic pain, MCP-1, RANTES, MIP-1α, fractalkine, SDF-1, peroxisome proliferator-activated receptors

INTRODUCTION

Chronic pain presents a serious medical problem. Current pain therapies show limited efficacy and many patients experience pain that is refractory to the available treatments. Neuropathic pain is frequently characterized by inflammation which can lead to sensitization in both the central and peripheral nervous systems. Key inflammatory mediators that are known to participate in chronic pain, including chemokines, have emerged as new therapeutic targets. Here, for the first time, we present a review of the literature linking chemokines in neuropathic pain to activation of peroxisome proliferator-activated receptors (PPARs). Ligand bound PPARs are known to inhibit the expression of inflammatory genes by a process termed *transrepression*. Among the genes repressed by activated PPARs are those of chemokines and their receptors. Early clinical trials indicate that PPAR agonists can be effective at alleviating neuropathic pain, even in patients who failed to respond to other treatments. While much remains to be understood about how PPAR agonists achieve this effect, it seems probable that inhibiting the expression of pain-causing inflammatory mediators like chemokines represents at least one mechanism for pain reduction.

NEUROPATHIC PAIN

Pain is defined as an unpleasant sensation induced by a noxious stimulus. There are two commonly used criteria for distinguishing acute from chronic pain. Acute pain is typically defined as pain associated with an injury and pain that is relatively short in duration. Chronic pain is sometimes defined as pain that persists beyond the expected healing time of an injury. Alternatively, researchers and clinicians may use arbitrary time points to define chronic pain as pain that persists beyond this time frame, e.g., 3 months. Acute pain serves an important function by warning individuals of tissue damage. Chronic pain, when it is dissociated from an injury, does not serve this purpose. Instead, chronic pain results from dysregulation, also called sensitization, of the nervous system. Persistent pain can produce permanent functional changes in the pain perception pathway. Sensitization can occur at all levels of the pain neuraxis, in both the central and peripheral nervous systems (Costigan et al., 2009).

Chronic pain can be divided into two classes, nociceptive and neuropathic. Nociceptive pain is caused by activation of nociceptors in the skin, tissue, or viscera in response to injury. Neuropathic pain results from damage to the somatosensory nervous system. Peripheral neuropathies may involve injured sensory,

motor, or autonomic nerves. In the central nervous system, injury, stroke, or disease in the brain or spinal cord can also generate a state of chronic, neuropathic pain. These causes of neuropathic pain often evoke a strong immune response (Woolf and Mannion, 1999; von Hehn et al., 2012).

INFLAMMATION

Animal models of neuropathic pain have illuminated some of the complex mechanisms that underlie the development and maintenance of pain states after injury. Researchers have been able to reproduce human-like pain responses in animals, and study the mechanisms that generate such pain behaviors as well as possible treatments. Neuropathic pain symptoms are often heterogeneous in nature, and animal models have shown that several mechanisms are likely involved. Mechanisms including neuronal hyperexcitability (Wall and Gutnick, 1974; Empl et al., 2001; Wu et al., 2002; Coull et al., 2005; Jung et al., 2008; Bedi et al., 2010), changes in gene expression (Plunkett et al., 2001; Barclay et al., 2002; Bhangoo et al., 2007; Sandhir et al., 2011), and alterations in the neuronal environment (Frisén et al., 1993; Sommer et al., 1993; Zelenka et al., 2005) not only contribute to neuropathic pain, but may also facilitate and enhance one another. Physical damage to the nervous system, as well as changes in chemical and electrical signals in and around neurons contributes to pain.

Inflammation is an adaptive response to bodily insults like infection and tissue injury. The immune system response to nerve injury alters the chemical environment of sensory and pain neurons. Evidence points to a role for immune cells and inflammatory mediators in generating not only inflammatory pain but chronic, neuropathic pain as well (Moalem and Tracey, 2006; Medzhitov, 2008).

Many inflammatory mediators have been implicated in cases of neuropathic pain, yet to what degree immune system actions specifically cause and/or maintain neuropathic pain is incompletely understood. Research in animal models supports the conclusion that neuroimmune signaling contributes to sensory dysregulation and neuropathic pain. At the most fundamental level, injured neurons and glia release inflammatory mediators that activate resident and recruit circulating immune cells. These cells then release cytokines and chemokines that can alter neuronal signaling (Calvo et al. (2012) have written a superior review on this topic).

TREATMENTS

Recent epidemiological studies have placed the prevalence of chronic, neuropathic pain at 6–8% in the general population (Torrance et al., 2006; Bouhassira et al., 2008). However, the occurrence of pain differs greatly between neuropathies. For example, the prevalence of neuropathic pain in spinal cord injury patients is between 25–60%; while 70–90% of patients suffering from Guillain-Barré Syndrome report neuropathic pain (Moulin, 1998; Werhagen et al., 2004). Symptoms are many and vary from patient to patient. Pain phenotypes are not always specific to a neuropathy, and pain can result from neuropathy as well as from medications taken to treat the condition (Nandi, 2012). Patients may present multiple pain phenomena simultaneously, and their pain phenotypes can change over time. These observations suggest that different mechanisms may be at play within a particular neuropathic condition and even within a single patient.

Several groups of drugs have been utilized in neuropathic pain treatment; among them are analgesics like opiates, anti-inflammatory drugs including steroids, tricyclic antidepressants, anticonvulsants, antiepileptics, antihypertensives, local anesthetics, sodium channel blockers, NMDA receptor antagonists, SSRIs (selective serotonin-reuptake inhibitors), and cannabinoids (Moulin, 1998; Pöllmann and Feneberg, 2008; Park and Moon, 2010; Nandi, 2012). Side effects are common, and the use of nearly all these medications is complicated by concerns about their safety and efficacy. Apprehensions about drug dependence, tolerance, and other side effects arise when drugs are used chronically, especially at increasing doses. In some cases, patients may benefit from a treatment for a time, suddenly stop responding, and require a new therapy. For the most extreme neuropathic pain conditions, drugs may incompletely treat pain or fail to do so altogether (Harden and Cohen, 2003). Drugs that are well tolerated and effective at treating the most severe pain have yet to be developed.

CHEMOKINES

Mediators, such as cytokines and chemokines, are vital messengers in the inflammatory process playing roles as both proinflammatory and anti-inflammatory/prorepair signals that act upon numerous target tissues. Cytokines and chemokines are capable of directly influencing nociceptive transmission at every level of the pain neuraxis (Myers et al., 2006).

Chemokines (the name is derived from their function as CHEMOtactic cytoKINES) are small signaling molecules that serve as inflammatory mediators. Chemokine ligands are grouped into four families based on their amino acid sequence: alpha (CXC), beta (CC), gamma (C), and delta (CX3C). These designations refer to the positions of two conserved cysteine residues near the peptide's n-terminus. Chemokines exert their functions by binding to a family of seven transmembrane g-protein coupled receptors (GPCRs), which are given names correlated to the ligands they bind.

Chemokines were first identified for their role in inflammation (Yoshimura et al., 1987). Chemokines are released by damaged cells and have a vital function in facilitating the migration of leukocytes to the lesioned area (Charo and Ransohoff, 2006; Savarin-Vuaillat and Ransohoff, 2007). However, researchers discovered that while diversification of chemokines and their receptors correlates with the development of a complex immune system, some chemokines predate the evolution of the immune system (Huising et al., 2003; DeVries et al., 2006). Specifically, SDF-1 (stromal cell derived factor 1; CXCL12) and its cognate receptor, CXCR4, are found in life forms without immune systems. Further, SDF-1 and CXCR4 are constitutively expressed when many chemokines are upregulated only during inflammation. This discovery prompted increased research into chemokines and their receptors. Now more than 50 chemokines and 20 receptors have been identified, and the known roles they play are more varied.

Chemokine signaling is important for immune system homeostasis (immune surveillance and immune cell maturation) as well as for inflammation. Chemokines also serve key functions in hematopoiesis, angiogenesis and neurodevelopment. Indeed, these roles are still observed in the adult, as SDF-1/CXCR4 signaling plays a role in adult neurogenesis (Lu et al., 2002) as well as generating tumor vasculature (Koshiba et al., 2000; Rempel et al., 2000). More recent research has also demonstrated that chemokines can be potent neuromodulators. They can regulate neurotransmitter release, alter ion channel activity, and even act as neurotransmitters themselves (Qin et al., 2005; White et al., 2005a; Zhang et al., 2005; Sun et al., 2006; Jung et al., 2008).

CHEMOKINE SIGNALING IN CHRONIC INFLAMMATION AND NEUROPATHIC PAIN

Chemokine expression is a downstream effect of the inflammatory cascade. Chemokine transcription is typically stimulated by "upstream cytokines" like interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα). The upregulation of IL-1β and TNFα by sensory neurons is a very early, post trauma event (Uçeyler et al., 2007; Sacerdote et al., 2008). Chemokines are capable of selectively recruiting monocytes, neutrophils, and lymphocytes, by establishing a chemical concentration gradient, or "chemokine gradient". Cells expressing cognate chemokine receptors travel this gradient toward the location of highest chemokine concentration. Chemokines not only act on their receptors to make immediate alterations to cell signaling but also activate the expression of further downstream inflammatory mediators.

Chemokines are expressed both as part of the normal inflammatory response and as part of the pathology of chronic inflammation. Chemokine signaling has been implicated in conditions ranging from autoimmune disorders to vascular and pulmonary diseases, transplant rejection, and cancer. In neurological diseases with an inflammatory component, such as multiple sclerosis, Alzheimer's disease and HIV-1 infection, research has shown that chemokines serve many key roles, including the generation and maintenance of disease associated neuropathic pain. Chemokine expression is also observed in many animal models of neuropathy induced pain.

Oh et al. (2001) made an important connection between chemokines and pain *in vivo* when they demonstrated that injection of SDF-1, RANTES, and MIP-1α could produce hindpaw tactile allodynia in rats. In neuroinflammation, chemokines are released not only by resident and recruited immune cells but also by damaged, inflamed nervous system cells. Further, neurons and glial cells that produce chemokines are also targeted by those same signals. DRG neurons in culture express chemokine receptors including CXCR4, CCR4, CCR5, and CX3CR1, the fractalkine receptor (Oh et al., 2001). Additionally, a subset of cultured DRG neurons demonstrated strong excitation in response to administration of chemokines including SDF-1, MCP-1, RANTES, and fractalkine (Oh et al., 2001; White et al., 2005b). Chemokines are coexpressed in neurons along with pain associated neurotransmitters including CGRP and substance P (Oh et al., 2001; Li et al., 2003; Dansereau et al., 2008). Excitation by chemokines, including CXCL1 and MCP-1, also prompt the release of CGRP, further strengthening the connection between chemokines and pain (Qin et al., 2005; Jung et al., 2008).

It is well known that chemokines and other proinflammatory mediators make a cytotoxic environment that strongly affects local cells (Frisén et al., 1993; Sommer et al., 1993). Further, chemokine upregulation can persist for weeks after injury in animal models (Flügel et al., 2001; Zhang and De Koninck, 2006; Bhangoo et al., 2007). Thus, persistent chemokine upregulation is not only consistent with a role in hypersensitizing nociceptors, but also provides an attractive therapeutic target.

TARGETING CHEMOKINE SIGNALING TO TREAT NEUROPATHIC PAIN

Several of the pain treatments described above, such as tricyclic antidepressants and NMDA receptor blockers, act primarily upon neuronal targets. As neuron-glial cell interactions have been recognized as fundamental to pain pathology, drugs that target messengers like cytokines and chemokines which signal between these different cells have drawn more attention. Several methods may be useful in disabling chemokine-receptor communication including antibodies and antagonists. Pharmaceutical companies have developed and tested antagonists to a number of cytokine and chemokine receptors with mixed results.

For example, CCR2 receptor antagonists (CCR2-RAs) are capable of temporarily relieving pain in some animal models when administered after the establishment of neuropathic pain. CCR2-RAs can block established pain for a matter of hours after injection in an lysophophatidylcholine (LPC) model (Bhangoo et al., 2007), a chronic constriction injury model (Serrano et al., 2010; Van Steenwinckel et al., 2011), a trigeminal pain model (Zhang et al., 2012), and a chemotherapy drug induced pain model (Pevida et al., 2013). A recent study by Padi et al. (2012) used a CCR2/CCR5 receptor antagonist to treat pain. They propose that a broad-spectrum chemokine receptor antagonist may be a more powerful therapy.

In spite of their promise, very little data has been published on the use of CCR2-RAs to treat pain in human neuropathy. Pease and Horuk (2009) describe CCR2-RAs in clinical trials for a variety of human disease conditions, not simply pain treatment (Pease and Horuk, 2009). Kalliomäki et al. (2013) published an inconclusive study using a novel CCR2-RA to treat post traumatic neuralgia, or pain following a traumatic event such as surgery, injection, and radiation. The study recruited test subjects with established pain and compared several pain measures taken before and after treatment. The researchers reported no significant improvement in pain symptoms on any measure between either drug group and placebo. However, they did show an increase in plasma MCP-1, and decreased monocyte levels suggesting that the antagonist had in fact acted upon its target. In the end the authors attributed their underwhelming results to tester variability, too many patient test centers, and a heterogeneous population of pain types and causes (Kalliomäki et al., 2013).

While antagonists are one important avenue of therapy, their limitations argue strongly for the development of drugs that can better block chemokine/receptor communication. A method for targeting chemokine signaling this way may be to limit the gene expression of the chemokine and/or receptor. As long-term

changes in gene expression underlie the persistent upregulation of chemokines in chronic pain, changes in a gene's transcriptional regulation may allow alterations of that gene's expression level. Thus, in order to counteract the harmful chemokine upregulation seen in chronic pain, targeting the regulatory elements of transcription may be fruitful.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

PPARs are a family of nuclear receptors which act as lipid activated transcription factors. This family consists of three different isoforms: PPARα, PPARβ/δ, and PPARγ. These three receptors have different tissue distributions and distinct biological roles. However, each can affect both positive and negative regulation of inflammatory and metabolic genes. PPARs are activated by both endogenous ligands and synthetic drugs. Endogenous agonists include unsaturated fatty acids, eicosanoids, prostaglandins, components of low density lipoproteins, and derivatives of linoleic acid. The most commonly used synthetic agonists for PPAR receptors include the fibrates, which bind PPARα the thiazolidinediones (TZDs), or glitazones, which bind PPARγ and the glitazars, which bind both.

Canonically, PPARs form heterodimers with retinoid X receptors (RXRs) and bind to peroxisome proliferator response elements (PPREs) located in the promoter region of target genes. When inactive, PPAR-RXR is bound to a corepressor complex. Ligand binding to PPARs induces a conformational change and the release of the corepressor complex for degradation. The activated heterodimer then recruits a coactivator complex which facilitates gene expression. In their capacity as metabolic regulators, PPARs modulate several vital cellular functions including adipocyte differentiation, fatty acid oxidation, and glucose metabolism.

Research in the last decade has outlined another important function of PPARs: the inhibition of inflammatory gene expression. A study published in *Nature* by Jiang et al. (1998) was the first to demonstrate that both natural and synthetic PPARγ agonists could block the production of proinflammatory cytokines, TNFα, IL-6, and IL-1β, in cultured monocytes. In the course of their study, the authors made the intriguing observation that the nature of the inflammatory agent used to induce cytokine expression in monocytes effected the outcome of the PPARγ agonist treatment. Specifically, 15d-PGJ_2 and troglitazone inhibited TNFα expression in monocytes stimulated by okadaic acid or phorbol ester but not lipopolysaccharide (LPS).

In the same issue of *Nature*, Ricote et al. (1998) presented evidence that activated macrophages upregulate PPARγ. They further demonstrated that ligand bound PPARγ inhibits inflammatory gene expression through a process termed *transrepression* by targeting specific transcription factors including NF-κB, AP-1, and STAT. Transrepression is any mechanism by which a nuclear receptor, when bound to a ligand, can repress gene expression by interaction with transcription factors and regulatory proteins, not by direct interaction with specific DNA sequences. There are several forms of transrepression, including histone modification, block of RNA polymerase hyperphosphorylation, coactivator complex disruption, coactivator complex competition, inhibition of corepressor clearance, etc. (Pascual and Glass, 2006).

PPAR FUNCTIONS IN INFLAMMATION

While PPARα and β/δ have pertinent anti-inflammatory effects, the role of PPARγ as a negative regulator of inflammatory genes, has been more completely explored. As outlined above, inactivated PPARγ-RXR binds to a corepressor complex at PPREs preventing gene expression. However, according to Christopher Glass and colleagues (Pascual et al., 2005), PPARγ is also capable of transrepressing inflammatory gene expression in macrophages by inhibiting corepressor clearance (**Figure 1**). Under basal conditions, corepressor complexes suppress inflammatory gene expression. In an inflammatory state, signaling through receptors such as toll-like receptors (TLRs) begins an inflammatory cascade. First, repressor complexes are ubiquinated and degraded. Next, inhibition of NF-κB is relieved and it translocates to the nucleus where it binds to the promoter region of target genes, initiating transcription.

However, ligand binding to PPARγ allows receptor SUMOylation, and this event directs PPARγ to a specific nuclear corepressor/histone deacetylase 3 complex (NCoR-HDAC3) bound to inflammatory gene promoter regions. SUMOylated PPARγ stabilizes this complex and prevents its degradation by blocking the recruitment of ubiquinylation/19 s proteosome machinery that is typically responsible for corepressor complex removal prior to gene transcription. Activated PPARγ maintains the NCoR portion of the complex in place thus keeping the target gene inactive (Pascual et al., 2005). This research provides one mechanistic explanation for PPARγ's change from gene activating to gene repressing.

Additional work by Wen et al. (2010) in mesangial cells of the kidney has outlined a separate mechanism by which unliganded and ligand bound PPARγ serve different functions in NF-κB pathway facilitated gene expression (**Figure 1**). They reported that PPARγ ligands, the natural agonist, 15d-PGJ_2, and synthetic molecules, troglitazone and ciglitazone, were able to block TNFα induced, NF-κB dependent expression of RANTES (CCL5) and MCP-1 (CCL2). They specifically explored the mechanism by which suppression of RANTES was achieved. The authors reported that downstream signalers of TNFα binding relieve inhibition of the p65 subunit of NF-κB by IκB, then phosphorylate p65, and induce its translocation to the nucleus. Once there, p65 binds to unliganded PPARγ, a relationship that is required for p65 to bind to its target κB site at the RANTES promoter and facilitate gene transcription. Yet, when PPARγ binds a ligand, due probably to a conformational change, PPARγ can no longer associate with p65. Under these conditions, p65 is not able to bind to κB sites, thus RANTES expression is transrepressed (Wen et al., 2010). Again, this mechanism provides another method by which PPARγ can alter its actions from promoting gene expression to actively repressing transcription.

These two models demonstrate that transrepression is complex and achieved by various mechanisms that are situationally-specific. Only a small part of this process as it is played out in different cell types under different conditions has been illuminated. While PPAR agonists may hold great therapeutic potential, their actions are many and varied. Within their capability are many positive effects, but also undesirable side effects that have unfortunately limited their use. Uncovering the actions of these drugs

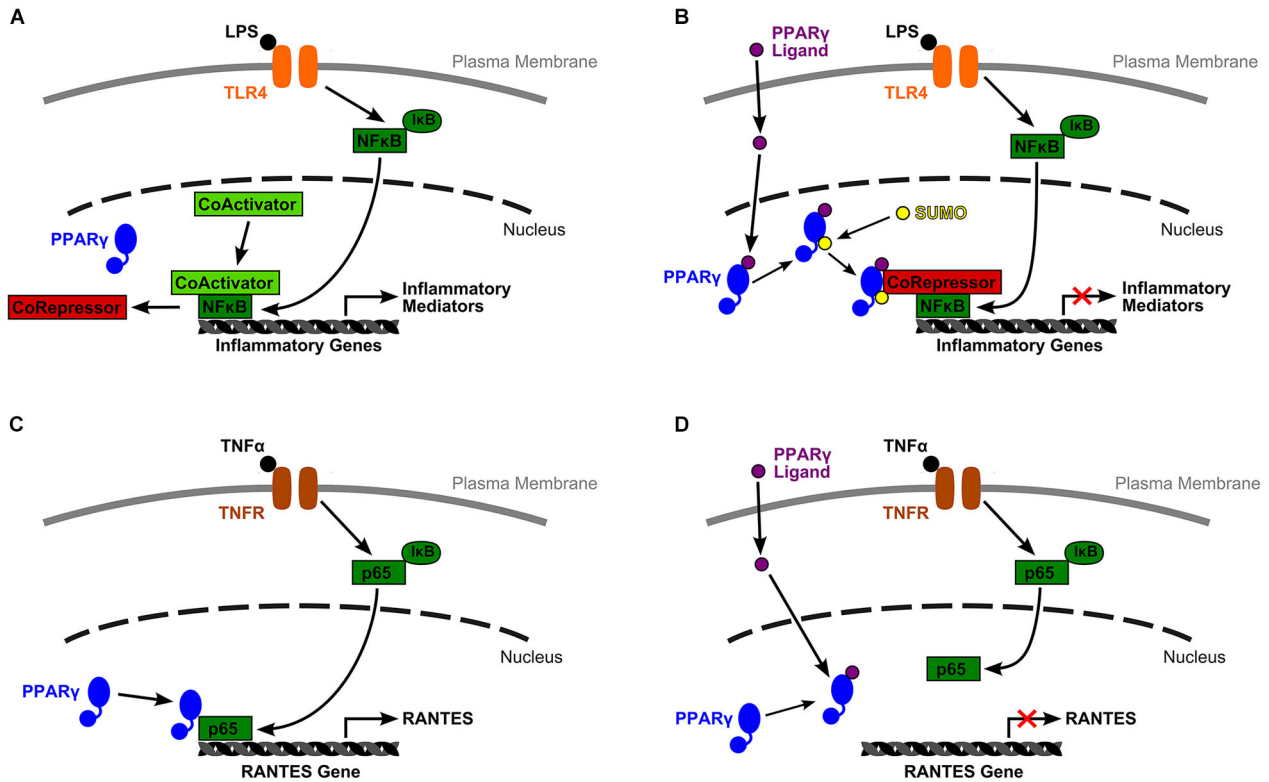

FIGURE 1 | Two models of PPARγ mediated inflammatory gene expression. (A) Under basal conditions, inflammatory gene expression is inhibited by a corepressor complex. An inflammatory signal, such as lipopolysaccharide (LPS) binding to TLR4, initiates an inflammatory cascade. Inhibition of NF-κB by IκB is lifted, and NF-κB translocates to the nucleus. The corepressor complex is removed for degradation while NF-κB recruits a coactivator complex, binds to the target gene's promoter, and initiates transcription. **(B)** Glass and colleagues (Pascual et al., 2005) proposed a mechanism by which activated PPARγ transrepresses inflammatory gene expression by inhibiting corepressor clearance. In their model, ligand binding to PPARγ allows receptor SUMOylation, which directs PPARγ to the NCoR-HDAC3 corepressor complex. PPARγ stabilizes this complex and prevents corepressor degradation, thus blocking gene transcription. **(C)** Wen et al. (2010) described a very different mechanism by which liganded and unliganded PPARγ have opposing effects on RANTES gene transcription. In their model, downstream TNFα inflammatory signals relieve NF-κB inhibition, phosphorylate the p65 subunit of NF-κB, and induce its nuclear translocation. There, unliganded PPARγ is *required* for successful association of p65 with the RANTES promoter. **(D)** However, ligand bound PPARγ is incapable of associating with p65, probably due to a conformational change, and RANTES expression is transrepressed.

sufficiently to separate their gene activating and gene repressing effects, inform more directed treatments, or even permit the development of "designer" pharmaceuticals whose side-effects are reduced will take significant further exploration (Glass and Saijo, 2010).

PPAR AGONISTS CAN ALTER CHEMOKINE EXPRESSION

A large number of studies have investigated the effects of PPAR agonist administration on inflammatory mediator expression in many tissues and disease models. There is significant evidence from models of diabetes, arthritis, atherosclerosis, Parkinson's disease, Alzheimer's disease and others that administration of PPAR natural ligands and synthetic agonists has anti-inflammatory effects. Specific reductions in proinflammatory chemokines and cytokines has been observed in numerous cells types: renal cells (Wang et al., 2011; Lu et al., 2013), vascular smooth muscle cells (Marchesi et al., 2013), adipocytes (Guri et al., 2008; Ueno et al., 2012), mesothelial cells (Sauter et al., 2012), epithelial cells (Neri et al., 2011), splenocytes (Bassaganya-Riera et al., 2011), monocytes/macrophages (Han et al., 2005; Tanaka et al., 2005; Hounoki et al., 2008; Liu et al., 2012), astrocytes (Lee et al., 2008, 2012), and microglia (Kim et al., 2012).

MCP-1/CCL2 EXPRESSION

As discussed above, signaling between monocyte chemoattractant protein-1 (MCP-1) and its cognate receptor, CCR2, has garnered a great deal of attention by researchers seeking to identify those chemokines that play the most important roles in neuroinflammation and neuropathic pain. MCP-1/CCR2 signaling has demonstrated some non-redundant effects, particularly in monocyte/macrophage recruitment, which make these two a most promising therapeutic target. For example, Abbadie et al. (2003) showed that CCR2-/- mice show a pain free phenotype after sciatic nerve ligation, a model of neuropathic pain, and a marked decrease in nociceptive

behavior after formalin injection, a model of inflammatory pain, when compared with controls. Further, MCP-1 and CCR2 remain upregulated for a long period after injury in several models. This evidence suggests that they serve a long-lasting function.

Information on PPARγ agonist induced inflammatory gene repression in nervous system cells types is limited. Real time PCR data on whole CNS tissue homogenate has shown suppression of MCP-1 expression by TZDs in an ischemic stroke model (Tureyen et al., 2007), a traumatic brain injury model (Yi et al., 2008), and a spinal cord injury model (Park et al., 2007). In the latter case, TZDs also conferred a number of neuroprotective effects (decreased lesion size, motor neuron loss, myelin loss, astrogliosis and microgliosis, and increased motor function recovery) via a PPARγ dependent mechanism.

An early study in Paul Drew's lab (Kielian et al., 2004) tested the effects of 15d-PGJ_2 effects on many cytokines and chemokines. In a model of brain bacterial infection, 15d-PGJ_2 reduced microglial expression of several proinflammatory cytokines including MCP-1. The group followed up with a series of parallel studies (Storer et al., 2005a,b; Xu et al., 2005) that tested the efficacy of endogenous and synthetic PPAR ligands on proinflammatory cytokine and chemokine inhibition in LPS stimulated cultured microglia and astrocytes. Both prostaglandin PPARγ agonists, 15d-PGJ_2 and PGA2, strongly inhibited MCP-1 production in microglia. Rosiglitazone also robustly decreased MCP-1 expression, but ciglitazone did so only at the highest tested doses, while pioglitazone had no effect. Astrocytes showed greater resistance to PPARγ agonist induced MCP-1 repression. PGA2 strongly inhibited MCP-1 upregulation while 15d-PGJ_2 had a modest improving effect. However, all the TZDs had an effect only at the very highest dose. Finally, fibrates, synthetic PPARα agonists, also blocked MCP-1 expression in microglia.

Like astrocytes and microglia, resident and circulating immune cells also play a large role in neuropathic pain. PPARγ is upregulated in macrophages during inflammation, and agonists can reduce the inflammatory migration, proliferation, infiltration, and phagocytotic ability of these cells (Ito et al., 2003; Tureyen et al., 2007; Hounoki et al., 2008; Liu et al., 2012). MCP-1/CCR2 signaling in macrophages is a target for PPARγ agonists. Treated monocytes/macrophages show decreased migration toward MCP-1 (Kintscher et al., 2000; Tanaka et al., 2005) and reduced MCP-1 expression (Rival et al., 2002).

Researchers have also reported that activated PPARβ/δ can repress MCP-1 expression in macrophages (Lee et al., 2003; Tan et al., 2005). Lee et al. (2003) reported a mechanism by which ligand bound and unliganded PPARβ/δ achieves differential regulation of MCP-1 expression in macrophages, which strongly echoes the mechanism for PPARγ regulation of RANTES expression described by Wen et al. (2010), above. Lee et al. revealed that the presence of PPARβ/δ in macrophages was associated with proinflammatory effects which were; however, completely blocked by the introduction of a PPARβ/δ agonist, GW501516. They suggested that unliganded PPARβ/δ interacts with other transcription factors to promote expression of MCP-1 and other proinflammatory cytokines.

CCR2 is also a target for activated PPARγ research shows that the two promoters which control CCR2 expression in monocytes are both subject to repression by ligand bound PPARγ (Chen et al., 2005). PPARγ agonists decrease infiltration by CCR2+ monocytes (Guri et al., 2008) likely by blocking CCR2 gene transcription (Tanaka et al., 2005). In one study, simvastatin, from the statin family of drugs used commonly for atherosclerosis management, was able to activate a peroxisome-proliferator response element in a PPARγ dependent manner to produce effects similar to those achieved by PPARγ agonists. Simvastatin treated monocytes failed to migrate toward MCP-1 probably because they had significantly decreased levels of CCR2 mRNA and protein (Han et al., 2005).

RANTES/CCL5 EXPRESSION

RANTES (regulated on activation, normal T cell expressed and secreted; CCL5) is another chemokine with a demonstrated role in pain behavior and sensitization. RANTES binds the CCR5 chemokine receptor which is known as an HIV-1 coreceptor. RANTES serves as a chemoattractant for memory T helper cells and leukocytes including blood monocytes and eosinophils. CCR5 expression on primary sensory neurons (Oh et al., 2001) has been demonstrated. RANTES delivery both in the periphery (Conti et al., 1998; Oh et al., 2001) and the central nervous system (Benamar et al., 2008) causes pain hypersensitivity. Finally, RANTES-/- mice show decreased nociceptive sensitivity and reduced macrophage recruitment after peripheral nerve injury (Liou et al., 2012). While more remains to be determined about the specific mechanisms by which RANTES participates in neuropathic pain, this chemokine clearly plays a role in peripheral sensitization.

In the case of RANTES, even less information exists than does for MCP-1 regarding the ability of PPAR agonists to alter its expression in nervous system cells. Only one such study has connected changes in PPAR signaling with a decrease in RANTES expression. Xiao et al. (2010) studied the effects of steroid receptor coactivator-3 (SRC-3) deficiency in experimental autoimmune encephalomyelitis (EAE) induced mice. SCR-3 is a p160 family coactivator that can transactivate nuclear receptors, including PPARs. They reported that SRC3-/- mice showed decreased disease severity and correlated a decrease in chemokine (RANTES, MCP-1, MIP-1α, and IP-10) expression with an increase in PPARβ/δ expression. The authors hypothesized that increased PPARβ/δ signaling altered the activation state of resident microglia, promoting an anti-inflammatory profile, as evidenced by an increase in IL-10 and other anti-inflammatory mediators (Xiao et al., 2010).

PPARγ agonists reduce RANTES expression in some immune cells as well. PPARγ activation blocks RANTES expression in immature dendritic cells (Szanto and Nagy, 2008). Interestingly, while prostaglandins reduce RANTES expression in LPS stimulated peritoneal macrophages, TZDs were unable to replicate this effect (Kim and Kim, 2007). The authors determined that 15d-PGJ_2 and PGA were acting via a PPARγ independent mechanism. While 15d-PGJ_2 altered RANTES expression in differentiated macrophages, it had no effect on either mRNA or protein levels of RANTES in peripheral blood monocytes, indicating

that differences in cell maturity constitute another situationally-specific outcome of drug administration.

RANTES is expressed in many other tissue types during inflammatory diseases. Animal models of inflammation in lung (Arnold and König, 2006), gastric (Cha et al., 2011), and renal (Li et al., 2005; Zhang et al., 2006; Wen et al., 2010) tissues show that PPARα and γ activation can reduce RANTES levels. As outlined above, Wen et al. (2010) described another transrepression mechanism by which liganded and unliganded PPARγ have opposing effects on RANTES expression through different interactions with the p65 subunit of NF-κB. Lastly, in human endometrial stromal cells, Pritts et al. (2002) demonstrated that rosiglitazone and 15d-PGJ_2 act at an upstream PPRE on the RANTES promoter to decrease the chemokine's transcription, showing that canonical PPARγ behavior may also have anti-inflammatory results.

MIP-1α/CCL3

MIP-1α (macrophage inflammatory protein-1α CCL3) is strongly upregulated throughout the pain neuraxis after nervous system injury. Increase in MIP-1α expression has been reported locally in Schwann cells and infiltrating macrophages after sciatic nerve injury (Kiguchi et al., 2010b) as well as in macrophages in the dorsal root ganglion (Kim et al., 2011). Both peripheral (Kiguchi et al., 2010a) and central (Knerlich-Lukoschus et al., 2011b) nervous system injuries cause upregulation of MIP-1α and it's receptor, CCR1, in the spinal cord. Traumatic spinal cord injury also increases the expression of MIP-1α and MCP-1 in the thalamus, hippocampus, and periaquaductal gray (Knerlich-Lukoschus et al., 2011a). Chemokine levels stay elevated for weeks after injury and MIP-1α/CCR1 expression correlates well with nociceptive behavior (Knerlich-Lukoschus et al., 2011b).

There is minimal data in the literature examining PPAR agonist modulation of MIP-1α expression in the nervous system. In one example of neuropathy, bacterial brain abscess, ciglitazone had neuroprotective and anti-inflammatory effects. Ciglitazone treatment decreased microgliosis overall, but increased phagocytotic activity by microglia. Additionally, protein levels of MIP-1α as well as other proinflammatory mediators (TNFα, IL-1β, and CXCL2) were decreased in the abscessed tissue (Kielian et al., 2004).

PPARγ signaling is also linked to decreased proinflammatory cytokine and chemokine expression in immune cells elsewhere in the body. Malur et al. (2009) demonstrated the importance of PPARγ expression in alveolar macrophages to maintain lung homeostasis. The authors reported that deletion of PPARγ in alveolar macrophages promoted a Th1 type inflammatory response including an upregulation of MIP-1α and IP-10. They proposed the use of PPARγ agonists for inflammatory lung diseases. However, an earlier study reported that 15d-PGJ_2 treatment enhanced lung inflammation caused by LPS in a mouse model. Instead of producing an anti-inflammatory response, 15d-PGJ_2 increased edema as well as proinflammatory chemokine (MIP-1α and MCP-1) and cytokine (IL-1β) expression.

A related study by Gosset et al. (2001) in mature dendritic cells showed that PPARγ activation yielded variable effects on chemokine expression depending upon the inflammatory agent employed. In once case, stimulation by a CD40 ligand, TZDs decreased the induced expression of MIP-1α as well as RANTES and IP-10. However, when LPS was used, TZDs had no effect on MIP-1α expression. This work, like that by Gurley et al. (2008) discussed below, demonstrates the situationally-specific nature of cellular responses to PPAR agonists.

FRACTALKINE/CX3CL1

Fractalkine, also designated CX3CL1 for the three amino acids that separate the characteristic N-terminal cysteines, is a unique chemokine. It is the only chemokine that can remain adhered to cells by means of a mucin-like stalk that tethers the chemokine domain to the plasma membrane. Cleavage by cathepsin S releases a soluble form of fractalkine (Clark et al., 2009). Fractalkine binds to CX3CR1, the fractalkine receptor, and is chemoattractive for T-cells and monocytes. Endothelial cells express the tethered form of fractalkine during inflammation. Its unique structure allows fractalkine to attract circulating leukocytes and assist in adhering them to the endothelium.

In chronic pain states, studies have shown a key role for fractalkine and the fractalkine receptor in microglial activation (Verge et al., 2004; Lindia et al., 2005; Yang et al., 2012). The fractalkine receptor is primarily expressed in microglia in pain related areas of the dorsal horn (Lindia et al., 2005). Intrathecal delivery of soluable fractalkine produces nociceptive behavior in animal models (Milligan et al., 2004; Zhuang et al., 2007). CX3CR1-/- mice show decreased neuropathic pain and microglial activation (Staniland et al., 2010).

In spite of abundant information about the role of fractalkine and its receptor in neuropathic pain, no studies have yet demonstrated the ability of any PPAR agonist to alter their expression in the nervous system. However, PPARγ activation has demonstrated ability to reduce fractalkine expression by inflamed endothelial cells as well as decreased fractalkine receptor expression on monocytes/macrophages (Imaizumi et al., 2002; Bursill et al., 2010; Wan and Evans, 2010). Barlic and Murphy (2007) reported that this PPARγ activation regulates a change in $CCR2^{hi}/CX3CR1^{low}$ monocytes promoting a change to $CCR2^{low}/CX3CR1^{hi}$ macrophages. Finally, Wan and Evans (2010) in their paper showing negative regulation of fractalkine receptor expression by rosiglitazone also demonstrated that an agonist to PPARβ/δ decreased fractalkine receptor expression albeit to a lesser extent than rosiglitazone.

Interestingly, there is evidence that fractalkine signaling may modulate PPARγ receptor expression. Mizutani et al. (2007) revealed that low levels of fractalkine/fractalkine receptor signaling promotes an increase in PPARγ expression, thus maintaining a low level of anti-inflammatory activity in intestinal macrophages. They point out that intestinal macrophages are, by necessity, hyporeactive to inflammatory stimuli. Similar to the relationship between PPARγ and MIP-1α in alveolar macrophages (Malur et al., 2009), these authors hypothesize that very low levels of fractalkine signaling help maintain intestinal homeostasis by modulating PPARγ expression.

SDF-1/CXCL12

SDF-1 (stromal cell derived factor-1; CXCL12) is an evolutionarily old chemokine that serves key functions in stem cell

migration and organ development for example in hematopoiesis, angiogenesis, and neurogenesis, as well as playing a part in inflammation. Along with other chemokines, peripheral administration of SDF-1 is pronociceptive (Oh et al., 2001). The SDF-1 receptor, CXCR4, is expressed in dorsal root ganglion neurons, and its expression is upregulated after peripheral nerve injury (Oh et al., 2001; Bhangoo et al., 2007). SDF-1 and CXCR4 expression is also upregulated in the spinal cord in a model of traumatic spinal cord injury (Knerlich-Lukoschus et al., 2011b). SDF-1/CXCR4 signaling has been implicated in HIV-1 associated pain; CXCR4 is a known HIV-1 coreceptor like CCR5 (Bhangoo et al., 2009). Finally, SDF-1/CXCR4 may also involved in mediating opioid induced neuropathic pain (Wilson et al., 2011).

A small body of evidence indicates that activated PPARγ signaling can block SDF-1/CXCR4 facilitated lymphocyte chemotaxis as well as decrease both chemokine and receptor expression. Walcher et al. (2008) demonstrated that PPARγ activation can, within minutes, reduce SDF-1 induced migration of CD4+ lymphocytes (Walcher et al., 2008). This suggests some immediate interference with an SDF-1 receptor, rather than any change in gene expression. However, PPARγ agonists have been shown to reduce SDF-1 expression in adipose tissue (Foryst-Ludwig et al., 2010) and aortic grafts (Onuta et al., 2007), both inflammatory disease models. Natural ligands and TZDs have reduced CXCR4 expression in tumor cells in a model of metastasizing cancer (Richard and Blay, 2008). The authors cited disruption of SDF-1/CXCR4 signaling in the metastasis of stem-like cancer cells by a PPARγ dependent mechanism as a possible new cancer control treatment.

PPARγ AGONIST ACTIONS MAY BE RECEPTOR DEPENDENT OR RECEPTOR INDEPENDENT

Although PPARγ agonists have proven able to reduce inflammatory gene expression, to what degree these agents require the PPARγ receptor to mediate their effects is still unclear. The evidence indicates that it is common for endogenous PPARγ ligands, particularly 15d-PGJ_2, to exert effects via PPARγ independent mechanisms. For example, Lee et al. (2008) demonstrated that when 15d-PGJ_2 decreases MCP-1 expression in INF-γ stimulated astrocytes it does so not by binding PPARγ but instead by modulating MAPK-phosphatase 1 (**Figure 2**). Many other studies have confirmed that at least some of the anti-inflammatory actions of 15d-PGJ_2 are PPARγ independent (Hounoki et al., 2008; Kim et al., 2012; Liu et al., 2012).

However, it is not only 15d-PGJ_2 that shows PPARγ independent activity. Welch et al. (2003) published data revealing that rosiglitazone utilizes two different mechanisms, depending upon its concentration, to alter proinflammatory gene expression in macrophages. Rosiglitazone inhibits production of LPS and INF-γ target genes via a PPARγ dependent mechanism at low doses, but at high doses it employs a PPARγ independent mechanism. The authors noted that the inhibition dose-response curve for rosiglitazone did not match its established binding affinity for PPARγ. So, using PPARγ-/- macrophages, they demonstrated that rosiglitazone still repressed proinflammatory genes and determined that rosiglitazone was binding to PPARβ/δ.

Finally, there is evidence that the effects of different PPARγ agonists may be a function of additional, modulatory signals. Gurley et al. (2008) demonstrated that pioglitazone and troglitazone could have varying effects in activated astrocytes depending upon the nature of a coadministered TLR ligand. They reported no change in MCP-1 expression after LPS (TLR4 ligand) and troglitazone. The same was true of single stranded RNA (TLR7/8 ligand) with troglitazone; yet ssRNA and pioglitazone facilitated an increase in MCP-1 expression. Most fascinating, when flagellin (TLR5 ligand) and pioglitazone were given, MCP-1 expression increased; however, when flagellin was accompanied by troglitazone, MCP-1 expression decreased.

From these data, we can gather that PPARγ agonist modes of action are complex, as are the variety of ways in which liganded PPARγ can facilitate either gene expression or transrepression. Further modification of activated PPARγ actions by other ligand-receptors and their intracellular signals, can also yield different results. Significant work remains to be done to elucidate such situationally-specific mechanisms in order to determine why some treatments work and others fail.

PPAR AGONISTS MODULATE NEUROPATHIC PAIN

As noted earlier, the use of PPAR agonists as a treatment has been explored in animal models of inflammation, brain injury, demyelination, and pain. The results of many of these studies are encouraging. PPAR agonists have been shown, in animal neuropathy models, to possess neuroprotective (decreased lesion volume), anti-inflammatory (decreased microglial activation and inflammatory gene expression), antiapoptotic (decreased number of apoptotic neurons), antioxidative, and neurologically improving effects (Drew et al., 2005; Zhao et al., 2005; Racke et al., 2006; Park et al., 2007; Costa et al., 2008; Yi et al., 2008; Di Cesare Mannelli et al., 2013). As the inflammation following neuropathy is strongly linked to the development of neuropathic pain states, it is reasonable to ask whether or not PPAR agonists can modulate neuropathic pain behavior in a manner similar to their anti-inflammatory effects.

USE IN HUMANS

Evidence from several clinical trials demonstrates that the endogenous PPARα agonist, palmitoylethanolamide (PEA), is an effective treatment for various human pain conditions. PEA was identified in 1957 as a fatty acid amide with anti-inflammatory properties (Kuehl et al., 1957). While PEA is a known agonist of PPARα, its anti-inflammatory effects may be mediated by additional receptors, including the other PPAR isoforms as well as TRPV1 and cannabinoid receptors. Further, PEA appears to have many possible target cells. Additional research is needed to expand our understanding of the mechanisms that underlie PEA's effects.

PEA is available in some European countries as a dietary supplement for medical purposes under the names Normast® and PeaPure® indicated for the treatment of pain and inflammation. It has demonstrated great efficacy in treating neuropathic pain, even in patients whose pain has proven refractory to other therapies (Biasiotta et al., 2010). Clinical trials have been conducted in patients with diabetic neuropathy (Schifilliti et al., 2014),

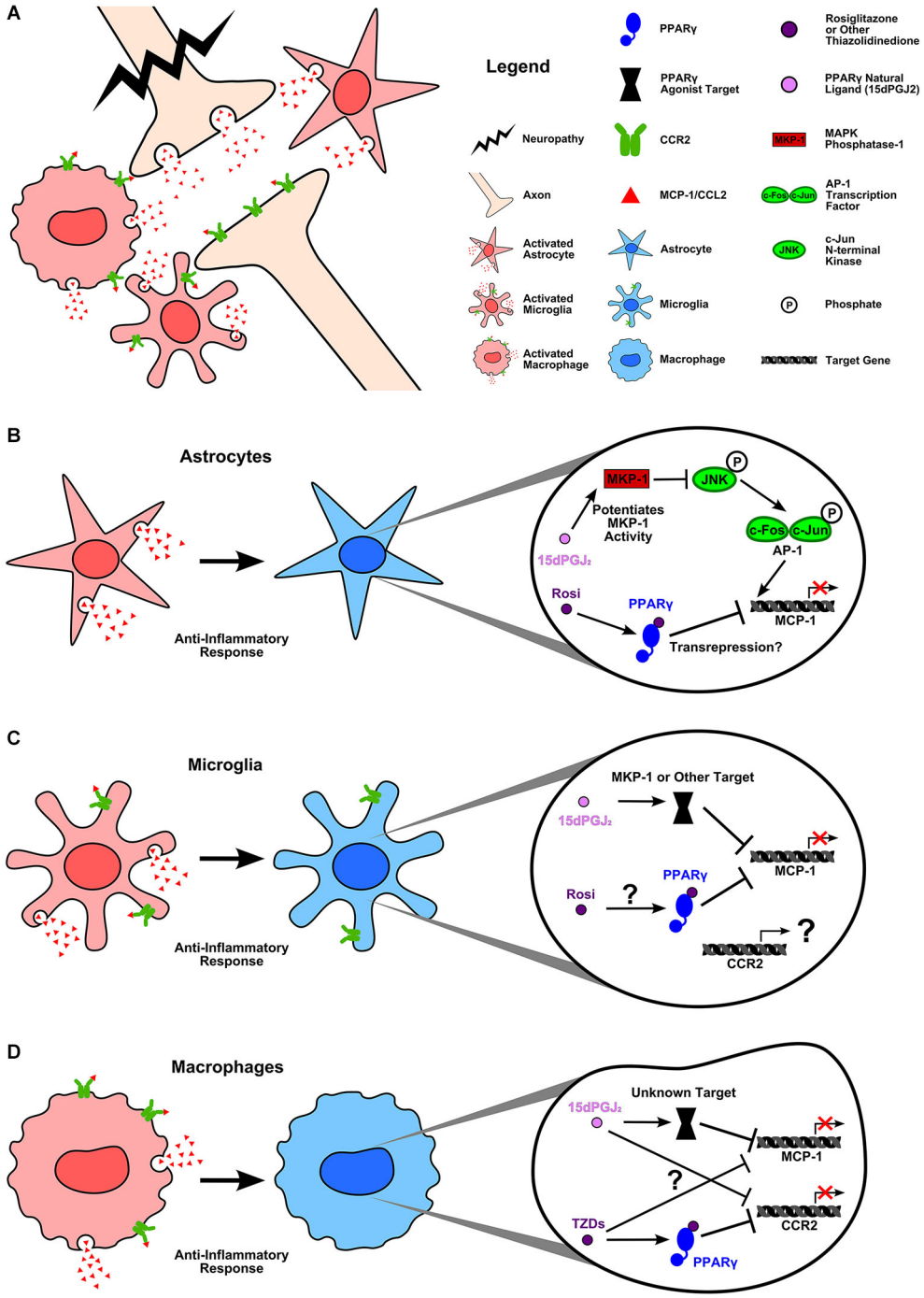

FIGURE 2 | PPARγ agonists inhibit MCP-1 and CCR2 expression in inflammatory neuropathy. **(A)** Damage to the central nervous system causes activation of astrocytes and resident microglia as well as recruited macrophages. Glial cells (Van Der Voorn et al., 1999; Abbadie et al., 2003; Yan et al., 2007; Zhang et al., 2007, 2012; Knerlich-Lukoschus et al., 2008) and macrophages as well as neurons (Zhang and De Koninck, 2006; Gao and Ji, 2010; Zhang et al., 2012) upregulate MCP-1 and CCR2 expression as part of the inflammatory response to injury. **(B)** Activated astrocytes express MCP-1, which can be blocked by rosiglitazone and 15d-PGJ_2. Lee et al. (2008) demonstrated that 15d-PGJ_2 inhibits INF-γ induced MCP-1 expression by potentiating the activity of MAPK phosphatase-1. MKP-1 targets JNK for dephosphorylation. This prevents the activation of the AP-1 transcription factor subunit, c-jun, thus inhibiting AP-1 mediated MCP-1 expression. In the case of rosiglitazone, it is unclear what mechanism is used to block MCP-1 expression; however, Lee et al. (2008) confirmed that rosiglitazone acts via PPARγ to inhibit INF-γ induced MCP-1. **(C)** Activated microglia upregulate MCP-1 and CCR2 during inflammation. Again, both rosiglitazone and 15d-PGJ_2 can block MCP-1 expression. While rosiglitazone's mechanism of action remains unclear, studies have verified that 15d-PGJ_2 is acting in a PPARγ independent manner (Lee et al., 2008; Kim et al., 2012). Lee et al. (2008) reported that, as in astrocytes, 15d-PGJ_2 acts upon MKP-1 to block

(*Continued*)

FIGURE 2 | Continued
INF-γ induced MCP-1 expression in microglia. No studies have yet examined the effects of natural or synthetic PPARγ agonists on CCR2 expression in activated microglia. **(D)** Recruited macrophages express both MCP-1 and CCR2. Thiazolidinediones (TZDs) decrease monocyte migration toward MCP-1 (Kintscher et al., 2000; Tanaka et al., 2005) likely by PPARγ dependent inhibition of CCR2 gene expression (Chen et al., 2005). However, whether or not TZDs act in a PPARγ dependent manner to block MCP-1 expression is unknown (Hounoki et al., 2008). In the case of 15d-PGJ_2, studies again indicate a PPARγ independent mechanism of action for decreasing LPS induced MCP-1 expression (Liu et al., 2012). 15d-PGJ_2 has a demonstrated ability to decrease CCR2 mRNA, yet the mechanistic target remains to be discovered (Tanaka et al., 2005). The ability of PPARγ agonists to decrease MCP-1 and CCR2 expression in cell types known to be involved in neuroinflammation and pain is encouraging. PPARγ agonists offer tantalizing hope of blocking proinflammatory chemokine signaling between glial cells, immune cells, and neurons which is known to be fundamental to neuropathic pain. However, these drugs have many and varied targets which complicates their use at present. Further research is needed to identify the mechanisms by which both natural and synthetic PPAR agonists reduce inflammation in the nervous system. Such knowledge will help researchers to identify the agonists best suited to preventing chronic inflammatory chemokine expression.

postoperative pain, sciatic pain, multiple sclerosis pain (Kopsky and Keppel Hesselink, 2012), chemotherapy pain (Truini et al., 2011), and post-stroke pain, among other conditions (Keppel Hesselink (2012) published a detailed review of studies using PEA to treat chronic pain).

Several characteristics of PEA make it a very attractive pain therapy. The first, mentioned above, is that it has been successful at reducing pain in patients whose conditions were either unaffected or incompletely treated by other medications. Second, both clinical trials and case studies have reported no side effects of PEA use. The lack of side effects has encouraged physicians to include PEA alongside more traditional pain medications such as oxycodone and pregabalin in a multimodal treatment plan. PEA has shown no drug-drug interactions when given with these medications. In fact, in several studies the addition of PEA to an existing treatment regimen has increased the therapeutic effectiveness and in some cases permitted a dose decrease of companion drugs. PEA has also been successful in combination with non-drug treatments such as physical therapy and acupuncture (Desio, 2010; Keppel Hesselink, 2012; Keppel Hesselink and Hekker, 2012; Kopsky and Keppel Hesselink, 2012; Schifilliti et al., 2014; Skaper et al., 2014).

Most recently, Sasso et al. (2013) published a study regarding a novel method for manipulating the anti-inflammatory and antinociceptive effects of PEA-PPARα signaling in animal models. These authors reported on a novel N-acylethanolamine acid amidase (NAAA) inhibitor, ARN077, which indirectly prevents the degradation of PEA. PEA is produced endogenously from precursors (fatty acid ethanolamides) by N-acyl-phosphatidylethanolamide phospholipase D as needed, and its levels are controlled by NAAA mediated hydrolysis. Sasso et al. reported that ARN077 attenuated neuropathic pain behavior by inhibiting NAAA activity and preserving PEA levels. Thus, maintaining PEA levels in injured tissues either by addition of exogenous PEA or preservation of endogenous PEA appears to be an effective pain treatment (Taylor, 2013). Indeed, if ARN077 were to prove an effective therapy in humans, it might serve well given in conjunction with Normast® or PeaPure®.

A NOTE ON THIAZOLIDINEDIONES

There is very little information regarding the use PPARγ agonists for neuropathic pain treatment in humans. In part, this is the result of conflicting data about the safety of key agonist, rosiglitazone. In 2007, Nissen and Wolski, published a meta-analysis of the cardiovascular side effects of rosiglitazone (Avandia®) treatment for type II diabetes mellitus. They concluded that rosiglitazone use was associated with an increased risk of myocardial infarction. In spite of a rebuttal publication by the RECORD (Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycaemia in Diabetes) study group (Home et al., 2007), the United States Food and Drug Administration (FDA) in 2010 imposed strong restrictions on rosiglitazone use in patients.

On November 25, 2013, the FDA delivered a press release announcing the removal of the majority of these restrictions on the prescription and use of Avandia after the final results of the RECORD clinical trial [NCT00379769] (Home et al., 2009) failed to uphold the findings of Nissen and Wolski.[1] The RECORD study results are a welcome development for rosiglitazone and other thiazolidinedione drugs which have shown such promise for treating diabetes and other conditions.

IN ANIMAL MODELS

Animal research has provided evidence that both natural and synthetic ligands to PPARα and PPARγ reduce pain. Agonists with demonstrated pain alleviating effects include the aforementioned rosiglitazone, pioglitazone, and 15d-PGJ_2 as well as PEA and fenofibrate. Other synthetic PPARα agonists, GW7647 and Wy14643, also reduce pain. While these results are very encouraging, there remains a major challenge in assessing the collective results of animal experiments. The wide variety of pain models, drugs, drug doses and schedules, drug administration routes, pain assessment methods, pain assessment timepoints, and limited investigation into the method(s) of drug action make the identification of unifying themes extremely difficult. However, some general conclusions can be drawn. The evidence indicates that *PPAR agonists modulate neuropathic pain in animal models*. . .

. . .*by acting at targets throughout the pain neuraxis*

The most potent PPAR agonist therapy requires repeated drug administrations beginning in the early phases of pain generation. It is logical that treatment will be more efficacious *before* the long-term changes underlying sensitization have been established. Yet, as dicussed above, PEA appears able to reduce even persistent pain in some clinical studies. Second, there is some confusion about the *in vivo* cellular targets of PPAR agonists. In some cases, different groups have published contradictory reports. Nevertheless, there is evidence that PPAR agonists can act to reduce pain at targets in the brain (D'Agostino et al., 2009; Morgenweck et al., 2010), in the spinal cord (Churi et al., 2008; Morgenweck et al., 2013), in the peripheral nervous system (LoVerme et al., 2006; Takahashi et al., 2011), and in the tissue (Hasegawa-Moriyama et al., 2012).

[1] www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm376516.htm

. . .*primarily via PPAR dependent mechanisms*

Wherever the location and cellular target(s) of PPAR agonists may be, the evidence points to PPARs as the primary mediators of pain alleviation by these agonists. In neuropathic pain models, researchers show that rosiglitazone (Park et al., 2007; Churi et al., 2008), pioglitazone (Park et al., 2007; Maeda et al., 2008; Jia et al., 2013; Morgenweck et al., 2013), and 15d-PGJ_2 (Churi et al., 2008) all act via PPARγ and PEA acts via PPARα (LoVerme et al., 2006; Di Cesare Mannelli et al., 2013). The same is true in models of inflammatory pain (D'Agostino et al., 2009) as well as of the neuroprotective effects (Park et al., 2007; Genovese et al., 2008) observed with these agents.

Yet, as dicussed earlier, PPAR agonists very clearly have receptor independent effects. Although pain studies have repeatedly verified the PPARγ dependent actions of rosiglitazone, it has been shown that, at high enough concentrations, rosiglitazone associates with PPARβ/δ (Welch et al., 2003). In another case, researchers used antagonists to PPARγ and PPARβ/δ to show that PEA, although not an agonist for either receptor, nevertheless appears to exert some downstream effect via these receptors (Paterniti et al., 2013). Others have tested the contribution of PPARγ and PPARβ/δ to the antinociceptive effects of PEA and found no association (LoVerme et al., 2006), thus further research is needed to definitively address these conflicting reports. Similarly, Costa et al. (2008) published their findings that PEA utilizes *not* PPARα, but instead interacts with cannabinoid receptor type 1 (CB_1), the transient receptor potential cation channel vanilloid receptor 1 (TRPV1), and PPARγ to reduce pain. Again, these results contradict the findings of other studies as mentioned above.

. . .*producing both changes in gene transcription and non-transcriptional effects*

Although the receptors involved in mediating the effects of PPAR agonists require further investigation, one downstream target of PPAR agonist signaling, NF-κB, has been clearly identified. Significant evidence shows that the results of PPAR agonist administration include block of IκB degradation, decreased p65 subunit phosphorylation, and a decrease in NF-κB translocation to the nucleus; the end result being a reduction in inflammatory gene expression (Dehmer et al., 2004; D'Agostino et al., 2007, 2009; Genovese et al., 2008).

However, research indicates that PPAR agonists have effects beyond those exerted upon transcription factors like NF-κB. Evidence shows that PPAR agonists, particularly rosiglitazone and PEA, can relieve pain rapidly but transiently (minutes-hours) (LoVerme et al., 2006; Churi et al., 2008; D'Agostino et al., 2009; Khasabova et al., 2012) as well as over the long-term (days) (Costa et al., 2008; Maeda et al., 2008; Jain et al., 2009; Takahashi et al., 2011; Jia et al., 2013). Thus, it seems clear that, in addition to effects that lead to modifications in gene transcription, these agonists must also have non-transcriptional targets. For example, LoVerme et al. (2006) reported that PEA administration resulted in a rapid decrease in the elecrophysiological response of spinal nociceptors to peripheral formalin injection.

. . .*ultimately altering the expression of inflammatory mediators including chemokines and their receptors*

While the mechanistic underpinnings PPAR agonist actions are known to be many and varied, the impact of these agents inhibitors of inflammation is well supported. Indeed, many studies have shown that PPAR agonists decrease the levels of upstream inflammatory cytokines known to induce chemokine expression, including TNFα, IL-1β, and IL-6 (Storer et al., 2005a,b; Park et al., 2007; Loría et al., 2008; Maeda et al., 2008; Impellizzeri et al., 2013; Jia et al., 2013; Paterniti et al., 2013).

In a few cases, specific decreases in chemokine expression have been reported in studies examining the effects of PPAR agonists on animal pain conditions. Impellizzeri et al. (2013) reported decreases in MIP-1α and MIP-2 levels after treatment with PEA and luteolin (an antioxidant) in a mouse model of rheumatoid arthritis. Park et al. (2007) demonstrated that pioglitazone decreased MCP-1 expression in spinal cord tissue in a model of traumatic spinal cord injury. Finally, Takahashi et al. (2011) observed a decrease in CCR2 expression in rosiglitazone-treated macrophages. In their study, the authors were able to achieve pain relief by transplanting these treated macrophages directly at the site of partial sciatic nerve ligation. It is possible that this result is part of a greater rosiglitazone effect on macrophages, as treatment with this drug seems to promote a polarity change from M1 (pro-inflammatory) to M2 (anti-inflammatory) (Hasegawa-Moriyama et al., 2012, 2013).

CONCLUSIONS

In the 15 years since the first reports that PPARγ serves functions in inflammation as well as metabolic regulation, researchers have opened the door on a subject of breathtaking complexity. In even these, earliest studies, investigators had begun to identify important questions about PPAR agonist actions that remain highly relevant today (Jiang et al., 1998; Ricote et al., 1998; Spiegelman, 1998).

The literature on PPAR signaling provides ample evidence that PPAR agonist administration can produce situationally-specific effects. These effects are the result, at least in part, of the ability of PPAR agonists to harness receptors other than PPARs, and to interact not only with transcription factors to impact gene expression but also to act at non-transcriptional targets to produce more rapid effects. To complicate matters further, the nature of those "situations" which generate different effects are not fully understood. In some cases, PPAR agonists known to bind to the same PPAR isoform, when administered under identical conditions can yield different results. Gurley et al. (2008) demonstrated this by showing that pioglitazone and troglitazone, both synthetic PPARγ agonists, produced opposite effects on flagellin induced MCP-1 expression. In other cases, agonists with the ability to act at the same PPAR isoform, achieve an identical effect by completely different mechanisms. For example, Lee et al. (2008) reported that rosiglitazone acted via a PPARγ dependent mechanism to decrease MCP-1 expression, while 15d-PGJ_2, which is a natural ligand for PPARγ nevertheless employed a PPARγ independent mechanism (MAPK signaling) to achieve the same result.

Research in animal models shows that disrupting the signaling of important inflammatory chemokines is sufficient to achieve pain relief. Yet, the results of efforts to translate these findings to effective pharmaceuticals have been disappointing. It has been speculated that redundancy in chemokine signaling prevents a specific chemokine receptor antagonist, for example, from proving clinically effective. The heterogeneous nature of neuropathic pain also presents a worrying medical problem. PPAR agonists have a demonstrated ability to alter the expression of chemokines, their receptors, and the upstream inflammatory cytokines typically responsible for stimulating chemokine expression. While, these broad-spectrum effects are potentially the key to the ability of PPAR agonists to reduce pain, they have also yielded some problematic side effects.

FUTURE DIRECTIONS

Given this prohibitive complexity, the question arises: why is it valuable to pursue greater understanding of PPAR agonists? There are two important reasons. The first is that these agents, both natural and synthetic, are extremely powerful. Continued investigation into how PPAR agonists achieve anti-inflammatory and antinociceptive effects is vital. Unlocking these mechanisms of action has the potential to inform new, safer, and more effective therapies. Second, these agonists are already being used effectively in clinical settings. Whether it be PeaPure® for pain management or Avandia® for insulin sensitization, PPAR agonists have clear, medical value which might yet be expanded if clinical trials using these agonists to treat conditions from cancer to dementia prove fruitful. PEA in particular has shown unprecedented potential to treat neuropathic pain. The apparent absence of side effects and drug interactions is very promising. Further, researchers and clinicians ought not overlook a treatment that has, even occasionally, proven effective where other therapies failed.

As stated earlier, Spiegelman (1998) identified two important questions raised by the works of Jiang et al. and Ricote et al. which remain relevant today. First, what underlies the situationally-specific outcomes of PPAR agonist treatment? For example, why do PPARγ agonists yield different results depending upon the particulars of the inflammatory response? Second, what are the targets acted upon by PPAR ligands when PPAR independent effects are seen? What are the relative contributions of PPARs vs. other targets to the various results of PPAR agonist treatment?

Concerning the particular effects of PPAR agonists on chemokine expression, there are additional questions and directions. First, PPAR agonists have a demonstrated ability to effect the expression of chemokines. More evidence is needed from pain models reporting the results of PPAR agonist treatment on chemokine expression in the nervous system in areas and cell types where chemokine signaling is known to contribute to pain. All PPAR isoforms are known to be expressed to some extent in parts of the central and peripheral nervous systems, although the literature has shown that their presence may not be required for some agonists to effect chemokine expression (Moreno et al., 2004; van Neerven and Mey, 2007; Maeda et al., 2008; Wang et al., 2012).

An additional question is: to what degree do PPAR agonists alter chemokine expression directly vs. altering the expression of upstream, inflammatory cytokines? There is abundant data demonstrating that PPAR agonists decrease the levels of cytokines such as TNFα, IL-1β, and IL-6 amongst others. This effect alone might be responsible for a concomitant decrease in chemokine expression. Yet, there is also evidence for direct action of ligand bound PPARs at chemokine promoters and other regulatory sites. Activated PPARs appear able to target RANTES expression both via "canonical" behavior and transrepression (Pritts et al., 2002; Wen et al., 2010). There is evidence for differential regulation of MCP-1 by activated PPARβ/δ (Lee et al., 2003). Finally, the promoters for CCR2, the receptor for MCP-1, are targets for activated PPARγ (Chen et al., 2005).

In conclusion, PPAR agonists are powerful agents with wide-ranging anti-inflammatory effects. Studies in animal models show these compounds have potent antinociceptive effects as well. Indeed, the PPARα agonist, PEA, has made a promising start as a treatment for human neuropathic pain conditions. Much work remains to be done to understand the complex mechanisms by which PPAR agonists achieve their anti-inflammatory and antinociceptive effects. However, the evidence to date shows that PPAR agonists reduce the expression of many inflammatory mediators, including specific chemokines that are known to generate and maintain chronic pain. We believe that PPAR agonists represent an exciting new way to manage chemokine expression in situations of neuroinflammation and pain.

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CXCL12 chemokine and GABA neurotransmitter systems crosstalk and their putative roles

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Since CXCL12 and its receptors, CXCR4 and CXCR7, have been found in the brain, the role of this chemokine has been expanded from chemoattractant in the immune system to neuromodulatory in the brain. Several pieces of evidence suggest that this chemokine system could crosstalk with the GABAergic system, known to be the main inhibitory neurotransmitter system in the brain. Indeed, GABA and CXCL12 as well as their receptors are colocalized in many cell types including neurons and there are several examples in which these two systems interact. Several mechanisms can be proposed to explain how these systems interact, including receptor–receptor interactions, crosstalk at the level of second messenger cascades, or direct pharmacological interactions, as GABA and $GABA_B$ receptor agonists/antagonists have been shown to be allosteric modulators of CXCR4. The interplay between CXCL12/CXCR4-CXCR7 and GABA/$GABA_A$-$GABA_B$ receptors systems could have many physiological implications in neurotransmission, cancer and inflammation. In addition, the $GABA_B$ agonist baclofen is currently used in medicine to treat *spasticity* in patients with spinal cord injury, cerebral palsy, traumatic brain injury, multiple sclerosis, and other disorders. More recently it has also been used in the treatment of alcohol dependence and withdrawal. The allosteric effects of this agent on CXCR4 could contribute to these beneficial effects or at the opposite, to its side effects.

Keywords: CXCL12/SDF1 chemokine, CXCR4, CXCR7, GABA, GABAA receptors

INTRODUCTION

The chemokine CXCL12/SDF1 has been found to play important roles in several processes involved in ischemic stroke and its' subsequent repair (Wang et al., 2012), brain tumor pathogenesis (Rempel et al., 2000; Duda et al., 2011), human immunodeficiency virus (HIV) encephalopathy (Li and Ransohoff, 2008), Multiple Sclerosis and stem cell migration (Carbajal et al., 2010). This chemokine of 67 amino-acids was first believed to act on a single receptor, the CXCR4. Since then, a second receptor has been found to be another target of CXCL12, namely CXCR7 (Schonemeier et al., 2008).

CXCR4 is a G protein-coupled receptor (GPCR) widely expressed in a variety of cell types including leucocytes, where it promotes migration, recruitment and activation (Bonavia et al., 2003; Salcedo and Oppenheim, 2003; Juarez et al., 2004; Choi and An, 2011; Comerford and McColl, 2011), neurons, where it modulates electrical activity (Banisadr et al., 2002; Guyon and Nahon, 2007; Rostene et al., 2011), and various cancers and metastases (Wang et al., 2006) where it is involved in tumor progression (Liu et al., 2006; Gao et al., 2010; Zhao et al., 2010). CXCR4 also binds the HIV-1 viral envelope glycoprotein gp120 (Doranz et al., 1997; Gabuzda and Wang, 2000). Thus CXCR4 is an important therapeutic target for stroke, inflammation, neuromodulation, cancer, and in the prevention of HIV infection. CXCR4 couples to the G_i family of proteins activating multiple G-protein dependent pathways (Lazarini et al., 2003; Busillo and Benovic, 2007). In neurons, CXCR4 stimulation has been shown to activate a G-protein-coupled inward rectifier K^+ (GIRK), a voltage-gated K channel Kv2.1 associated to neuronal survival, and to increase high voltage activated (HVA) Ca^{2+} currents (Guyon and Nahon, 2007; Shepherd et al., 2012).

CXCR7, contrary to CXCR4, could not be demonstrated to be coupled to G proteins. Despite its phylogenic relation and ligand binding properties, CXCR7 does not mediate typical chemokine receptor responses such as leukocyte trafficking. It was first believed to be mainly involved in ligand sequestration (Thelen and Thelen, 2008). However, recent studies show that ligand binding to CXCR7 activates MAP kinases through Beta-arrestins (Zabel et al., 2009; Rajagopal et al., 2010), and its functions could include modulation of circadian glucocorticoid oscillation and emotional behavior (Ikeda et al., 2013).

γ-aminobutyric acid (GABA) is the chief neuro-inhibitory neurotransmitter in mammalian systems but it also plays important roles in CNS development by regulating neurogenesis and synaptogenesis (LoTurco et al., 1995; Somogyi et al., 1995). In contrast to its inhibitory actions on adult neurons, GABA is capable of depolarizing neuronal progenitor cells and immature neurons (Ben-Ari, 2002; Rheims et al., 2008) and participates in the formation of a primitive network-driven pattern of electrical activity called giant depolarizing potentials (GDPs), which are critical for the generation of large oscillations of intracellular calcium, for activity-dependent modulation of neuronal growth and synapse formation (Ben-Ari, 2002). HIV-1 gp120, which binds and stimulates CXCR4, enhances GDPs in neonatal rat hippocampus (Kasyanov et al., 2006), underlying the role played by CXCR4 in the developmental process. Moreover, the developmental function

of GABA is in part regulated by GABA production, a process mediated by glutamic acid decarboxylases (GADs), the key rate-limiting enzymes for synthesis of GABA. Two GAD isoforms, GAD65, and GAD67, are expressed in the adult nervous system (Erlander et al., 1991). It has been shown that CXCL12/CXCR4 signaling induces expression of GAD67 in embryonic hippocampal cultured neurons via ERKs and the transcription factor Egr1, a mechanism which may promote the maturation of GABAergic neurons during development (Luo et al., 2008).

The GABA type A ($GABA_A$) receptors are ionotropic receptors. In response to binding GABA, their chloride-selective pore open resulting in hyperpolarization of the neuron. This causes an inhibitory effect on neurotransmission by diminishing the chance of a successful action potential occurring. The protein contains a number of different allosteric binding sites which modulate the activity of the receptor indirectly and are the targets of various other drugs, including the benzodiazepines, barbiturates, ethanol, neuroactive steroids, inhaled anesthetics, and picrotoxin, among others (Olsen and Sieghart, 2009). $GABA_A$ receptors are largely expressed in the nervous system but to a limited extent they can be found in non-neuronal tissues (Mohler et al., 1995).

Like chemokine receptors, $GABA_B$ receptors are GPCRs. $GABA_B$ receptors are obligatory heterodimers with 2 homologous subunits (GB_1 and GB_2) required for functioning (Bowery et al., 1980), are widely expressed and distributed in the central nervous system (Kaupmann et al., 1998) where they can activate the GIRK channel, negatively modulate HVA Ca^{2+} channels and activate diverse intracellular pathways (Guyon and Leresche, 1995; Laviv et al., 2011). $GABA_B$ receptors are also expressed on cells of the immune system with a possible link to the inflammatory response (Tian et al., 2004; Rane et al., 2005). As a consequence, there is a rich pharmacology aimed at targeting $GABA_B$ receptors, with numerous compounds currently being used with the presumption that they are highly selective for these receptors (Bowery, 1993; Froestl, 2010).

CO-LOCALIZATION OF CXCL12/CXCR4-CXCR7 AND GABA/GABA RECEPTOR SYSTEMS

In the periphery, CXCR4 and GABA receptors are often colocalized in the same cells. For example, in pathological conditions, CXCR4 and $GABA_A$ receptors are both expressed in leukocytes (Light et al., 2013) and CXCR4 and $GABA_B$ receptors are both found in cells of the immune system with a possible link to the inflammatory response (Tian et al., 2004; Rane et al., 2005; Wang et al., 2008). In the brain, CXCL12 receptors have been found to be expressed in several neuronal populations, which all express also GABA receptors (Banisadr et al., 2002; Schonemeier et al., 2008).

In the developing mouse CNS, expression of CXCR4 starts as early as embryonic day 8.5 and is sustained until adulthood. From E 15.5, both CXCR4 and CXCL12 are expressed in the cortex, olfactory bulb, hippocampus, as well as the meninges and endothelia. During postnatal development, CXCL12 influences the migration of GABAergic interneurons in the cortex by acting via CXCR4 (Stumm et al., 2003; Tiveron et al., 2006). In adults, CXCR4 immunoreactivity has been reported in many brain areas including cerebral cortex, caudate putamen, globus pallidus, substantia innominata, supraoptic, and paraventricular hypothalamic nuclei, ventromedial thalamic nucleus and substantia nigra, and in virtually all CNS cells including neurons, astrocytes, microglia, ologidendrocytes, and endothelial cells (Banisadr et al., 2002).

CXCR7, at embryonic stages, is distributed in the germinative zone of the ganglionic eminences, caudate putamen, and along the routes of GABAergic precursors migrating toward the cortex (Schonemeier et al., 2008). In the cortex, CXCR7 is expressed in GABAergic precursors and in some reelin-expressing Cajal-Retzius cells. Unlike CXCR4, CXCR7 is abundant in neurons forming the cortical plate and sparse in the developing dentate gyrus and cerebellar external germinal layer. CXCR7 is often co-localized with GAD in the postnatal cortex, hippocampus and cerebellum (Schonemeier et al., 2008). In the adult brain, CXCR7 is expressed by blood vessels, pyramidal cells in CA3, and mature dentate gyrus granule cells, which is reminiscent of the SDF-1 pattern. Further neuronal structures expressing CXCR7 include the olfactory bulb, accumbens shell, supraoptic and ventromedial hypothalamic nuclei, medial thalamus, and brain stem motor nuclei (Schonemeier et al., 2008).

Moreover, at the sub-cellular level, CXCL12 has partly a vesicular localization in axonal terminals (Reaux-Le Goazigo et al., 2012) and CXCR4 receptors are mainly located on the neuronal plasma membrane, where, like GABA receptors, they are present at presynaptic and post-synaptic sites of central terminals (Reaux-Le Goazigo et al., 2012).

Therefore, in the brain, the interactions between the two systems are made possible by a high level of colocalization.

EXAMPLES OF INTERPLAY BETWEEN THE TWO SYSTEMS

CXCL12 and GABAergic agents have complementary functionality. Similarly to CXCL12, GABA, and GABAergic agents have chemotaxic properties. For example, neutrophils (Rane et al., 2005) but also stem/progenitor cells (Zangiacomi et al., 2009) and embryonic neurons (Behar et al., 1996) and are attracted by GABA. GABAergic agents have also been shown to have anti-inflammatory properties[45]. The involvement of GABA receptors has been proposed in these effects, but curiously, the putative cross-talk between the two systems has been poorly investigated.

However, several groups have described the importance of the interplay between CXCR4 and $GABA_B$ receptors. For example, we and others have shown inhibition of CXCL12-induced migration of cancer cells by $GABA_B$ ligands (Wang et al., 2008; Guyon et al., 2013). Recently, it has also been shown that CXCL12 and GABA acting on its $GABA_A$ receptors interact to regulate axophilic migration of GnRH neurons (Casoni et al., 2012). GABAergic and CXCL12/CXCR4 systems interact, promoting linear rather than random movement. The simultaneous activation of these signaling pathways result in tight control of cellular speed and improved directionality along the migratory pathway of GnRH neurons (Casoni et al., 2012).

There is also evidence that CXCL12 can interact with GABA systems to modulate neurotransmission. Indeed, CXCL12 increases GABA neurotransmitter release in brain slices from different brain areas (Guyon et al., 2006; Heinisch and Kirby, 2010). Finally, agents acting on GABA receptors including GABA itself and $GABA_B$

receptors agonists/antagonists have been shown to reduce the effect induced by the activation of the CXCR4 receptor on calcium currents in brain slices of substantia nigra (Guyon et al., 2013).

PUTATIVE MECHANISMS OF INTERACTION (Figure 1)

INTERACTIONS BETWEEN RECEPTORS

Although CXCR4 is also functional as a monomer (Paavola et al., 1998; Veldkamp et al., 2008, 2009), it has been shown to homo-dimerize following CXCL12 interaction, a homo-dimerization which is necessary for its functionality and signaling (Mellado et al., 2001; Toth et al., 2004), and is accompanied by receptor phosphorylation as well as changes in signal transduction processes (Rodriguez-Frade et al., 2001). This enables the activation of the JAK/STAT pathway which allows the subsequent triggering of G-protein dependent signaling events (Vila-Coro et al., 1999). CXCR4 can also form heterodimers with other GPCRs. For example, CXCR4 has been shown to form heterodimers with CXCR7, CCR2, and CCR5 and delta opioid receptors (Percher-ancier et al., 2005; Pello et al., 2008; Levoye et al., 2009; Sohy et al., 2009). It is therefore tempting to imagine that CXCR4 could form heterodimers with $GABA_B$ receptors, which could explain the functional interactions that have been observed. However, although this has not been investigated in mammalian cell membranes, by co-expressing $GABA_B$ receptors tagged with Td tomato (red fluorophore) and CXCR4 receptors tagged with GFP (green) in the membrane of *Xenopus* oocytes, data obtained using TIRF

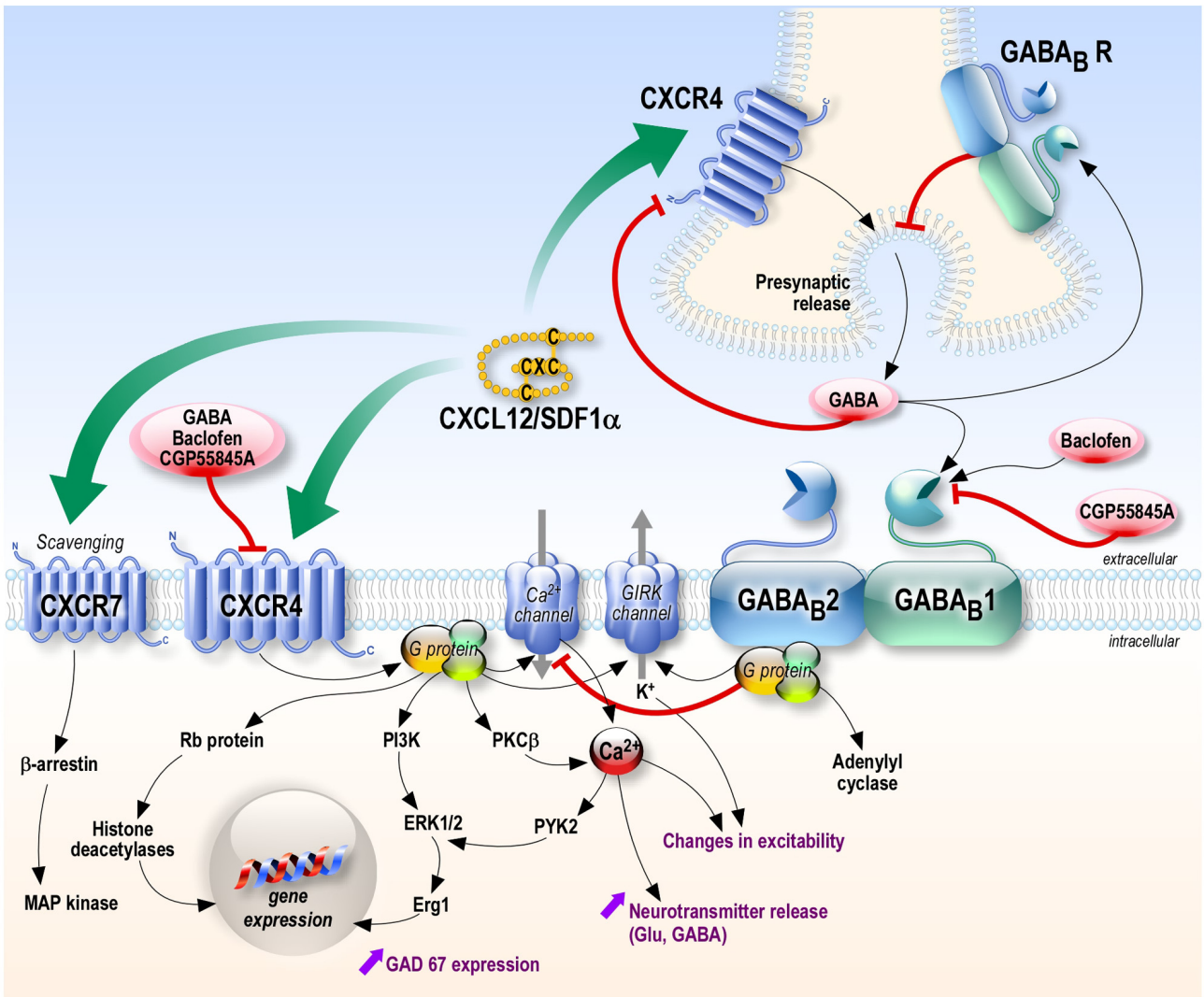

FIGURE 1 | Putative crosstalk between CXCL12-CXCR4/CXCR7 and GABAergic systems. The chemokine CXCL12 can act on its receptors CXCR4 and CXCR7, activating several intracellular pathways. At the pre-synaptic level, CXCR4 stimulation increases the pre-synaptic release of neurotransmitter such as GABA (opposite to $GABA_B$ receptors stimulation). CXCL12 receptors and $GABA_B$ receptors could interact at different levels including the receptors themselves (by heteromerization), the G-proteins they activate, their common target channels (G-protein activated inward rectifier potassium channels and voltage-activated calcium channels) or the second messenger cascades. In addition, GABA is a negative allosteric modulator of CXCR4 receptors, which could contribute to the negative feedback of GABA on its presynaptic release. Black and green arrows: activation; red lines: inhibition.

microscopy showed that CXCR4 and $GABA_B$ receptors did not co-localize in the membrane (Guyon et al., 2013), thus it is unlikely that these two GPCR receptors form heterodimers.

CROSSTALK AT THE LEVEL OF SECOND MESSENGER CASCADES

CXCR4 and $GABA_B$ receptors are both GPCR activating GIRK, and modulating voltage-gated channels such as K channels Kv2.1 and HVA Ca^{2+} currents (Guyon and Nahon, 2007; Shepherd et al., 2012), $GABA_B$ receptors stimulation decreasing HVA Ca^{2+} currents (Guyon and Leresche, 1995) while CXCR4 stimulation potentiating them (Guyon et al., 2008). Therefore, it is likely that the two systems might interfere at the level of the G protein, the second messenger cascade and/or the target channel in their action on neuronal excitability.

DIRECT PHARMACOLOGICAL ACTION

While somewhat unexpected, GABA and the agonists/antagonists of $GABA_B$ receptors (i.e., baclofen and the antagonists CGP55845 and 54626) were recently found to act pharmacologically directly on CXCR4 in an allosteric manner. Using electrophysiology in Xenopus oocytes and human embryonic kidney (HEK293) cells in which *Rat* CXCR4 and the GIRK channel were co-expressed, it could be demonstrated that $GABA_B$ antagonist and agonist modify the CXCL12-evoked activation of GIRK channels (Guyon et al., 2013). By expressing CXCR4 receptors in heterologous systems lacking $GABA_B$ receptors and performing competition binding experiments it could be investigated whether $GABA_B$ ligands bind to CXCR4. Electrophysiology data and FRET experiments suggested that $GABA_B$ ligands do not bind CXCR4 at the CXCL12 binding pocket suggesting allosteric modulation (Guyon et al., 2013). Finally, backscattering interferometry (BSI) on lipoparticles containing only the CXCR4 receptor allowed to quantify the CXCR4 binding affinities for the $GABA_B$ ligands (including GABA), which were in a similar range to the affinities of the ligands for $GABA_B$ receptors themselves, thus confirming that GABA and $GABA_B$ receptor ligands directly interact allosterically with the CXCR4 receptor (Guyon et al., 2013). In the future, it will be of interest to search for putative effects of GABA and $GABA_B$ receptor ligands on CXCR7.

PHYSIOLOGICAL CONSEQUENCES

There are many pathways by which GABA and CXCL12 systems can interact. GABA is able to block the effect of CXCL12 on CXCR4. Thus, it is likely that when the GABAergic system is activated, GABA released in the brain will antagonize the effect of CXCL12 on its receptor CXCR4, and thus could influence the chemokine neurotransmission as well as the inflammatory response in the central nervous system. Conversely, it has previously been shown that CXCR4 stimulation by CXCL12 can increase GABA release (Guyon and Nahon, 2007; Bhattacharyya et al., 2008; Qu et al., 2008). Therefore, there is reciprocal cross talk between these two systems that may affect several physiological levels.

NEUROTRANSMISSION

CXCR4 activation by CXCL12 has been shown to increase presynaptic neurotransmitter release and particularly GABA release in several neuronal populations (Guyon and Nahon, 2007; Bhattacharyya et al., 2008; Qu et al., 2008). If GABA can in turn block the effects of CXCL12, this could represent a negative feedback loop for presynaptic chemokine release (Guyon and Nahon, 2007; Bhattacharyya et al., 2008; Qu et al., 2008). Indeed, when applying CXCL12 for several minutes, a transient increase in the frequency of sPSCs is frequently observed, followed by a reduced activity (see Figure 3 in Guyon et al., 2006). This reduction could be due to an antagonistic effect of GABA, although desensitization of CXCR4 itself cannot be excluded.

In dopaminergic neurons of the rat substantia nigra, CXCR4 stimulation by CXCL12 induces an increase of release of presynaptic neurotransmitter, particularly of GABA (Guyon et al., 2006). CGP55845A (500 nM) blocks the outward GIRK current induced by CXCL12 (recorded in the presence of glutamate receptor blockers), which was first interpreted as an effect mediated through $GABA_B$ receptor stimulation by GABA spilling over following CXCL12 presynaptic stimulation and increase in $GABA_B$ release. However, GIRK currents might have been activated by the stimulation of postsynaptic CXCR4 by CXCL12, which was then blocked by CGP55845A.

INFLAMMATORY RESPONSE

Expression of $GABA_B$ receptors on cells of the immune system has recently been described, as well as a possible link to the inflammatory response (Tian et al., 2004; Rane et al., 2005). Along this line, it has been shown that baclofen, a selective $GABA_B$ receptor agonist, reduces chemotaxis from human mononuclear cells toward CXCL12 (Duthey et al., 2010). Given that human mononuclear cells express both $GABA_B$ and CXCR4 receptors, the finding that an agonist of one receptor alters the response to an agonist of the other receptor was interpreted to indicate a heterologous desensitization between chemokine and $GABA_B$ receptors. This observation along with our own observations on the chemotaxis of cancer cell lines expressing CXCR4 can also be reinterpreted as a direct allosteric action of baclofen on CXCR4.

PUTATIVE APPLICATIONS IN CANCER TREATMENT

Baclofen treatment was demonstrated to reduce the incidence of some carcinogen-induced gastrointestinal cancers in rats (Tatsuta et al., 1990) as well as human hepatocarcinoma cell growth (Wang et al., 2008). By contrast, baclofen promotes human prostate cancer cell migration (Azuma et al., 2003).

Similarly, it has been shown that GABA can affect the cell proliferation and have anti-inflammatory properties through inhibition of fibroblast proliferation, although the mechanism of action of GABA was not elucidated (Han et al., 2007). We suggest that GABA could have acted through the CXCR4 receptor, as CXCR4 is expressed on fibroblasts (Qu et al., 2008).

HIV INFECTION

Fusion of HIV-1 with the host cell membrane is initiated by the binding of the viral envelope glycoprotein gp120 to both the CD4 cell surface receptor and one of the CXCR4 or CCR5 chemokine receptors (Doranz et al., 1997; Gabuzda and Wang, 2000). It has therefore been suggested that the CXCR4-CXCL12 axis may be an important therapeutic target for prevention of HIV infection.

It will therefore be of interest to test the aptitude of baclofen and other $GABA_B$ receptor agents to affect the CXCR4–GP-120 interaction.

As a conclusion, agents interacting at CXCR4 could be useful to treat cancer as well as HIV infection. Baclofen is currently approved for the treatment of *spasticity* in patients with spinal cord injury, cerebral palsy, traumatic brain injury, multiple sclerosis and other disorders (Plassat et al., 2004; Guglani and Lodha, 2007; Kolaski and Logan, 2008; Rekand and Gronning, 2011). Recently, it has been used in the treatment of alcohol dependence and withdrawal (Addolorato et al., 2006). The allosteric effects of baclofen on CXCR4 could contribute to its beneficial effects as CXCR4 often co-localizes with $GABA_B$ receptors. At the opposite, it could be responsible for its side effects. Overall, the effect of GABAergic agents on CXCR4 suggests new therapeutic potentials for neurological and immune diseases.

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CXCL12 modulation of CXCR4 and CXCR7 activity in human glioblastoma stem-like cells and regulation of the tumor microenvironment

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Chemokines are crucial autocrine and paracrine players in tumor development. In particular, CXCL12, through its receptors CXCR4 and CXCR7, affects tumor progression by controlling cancer cell survival, proliferation and migration, and, indirectly, *via* angiogenesis or recruiting immune cells. Glioblastoma (GBM) is the most prevalent primary malignant brain tumor in adults and despite current multimodal therapies it remains almost incurable. The aggressive and recurrent phenotype of GBM is ascribed to high growth rate, invasiveness to normal brain, marked angiogenesis, ability to escape the immune system and resistance to standard of care therapies. Tumor molecular and cellular heterogeneity severely hinders GBM therapeutic improvement. In particular, a subpopulation of chemo- and radio-therapy resistant tumorigenic cancer stem–like cells (CSCs) is believed to be the main responsible for tumor cell dissemination to the brain. GBM cells display heterogeneous expression levels of CXCR4 and CXCR7 that are overexpressed in CSCs, representing a molecular correlate for the invasive potential of GBM. The microenvironment contribution in GBM development is increasingly emphasized. An interplay exists between CSCs, differentiated GBM cells, and the microenvironment, mainly through secreted chemokines (e.g., CXCL12) causing recruitment of fibroblasts, endothelial, mesenchymal and inflammatory cells to the tumor, *via* specific receptors such as CXCR4. This review covers recent developments on the role of CXCL12/CXCR4–CXCR7 networks in GBM progression and the potential translational impact of their targeting. The biological and molecular understanding of the heterogeneous GBM cell behavior, phenotype and signaling is still limited. Progress in the identification of chemokine-dependent mechanisms that affect GBM cell survival, trafficking and chemo-attractive functions, opens new perspectives for development of more specific therapeutic approaches that include chemokine-based drugs.

Keywords: CXCL12, CXCR4, CXCR7, glioblastoma, cancer stem cells

BACKGROUND

Chemokines (CKs) and their cognate receptors are constitutively expressed in the central nervous system (CNS) where they control complex physiological functions. In particular, the pleiotropic chemokine CXCL12 (formerly known as stromal-cell derived factor-alpha, SDF1-α) and its receptors CXCR4 and CXCR7 are key regulators in CNS development, and are involved in neuromodulation, neuroprotection, and control the interactions between neurons, microglia and astrocytes in adult brain (Rostene et al., 2007). Altered expression of CXCR4 and CXCL12 has also been associated to CNS diseases, such as HIV encephalopathy, stroke and multiple sclerosis, among others (Bajetto et al., 2001a; Rostene et al., 2011). A role for CXCL12 modulation of CXCR4 and CXCR7 was identified for most of the brain tumors including gliomas, meningiomas and even pituitary adenomas that frequently overexpress these receptors (Bajetto et al., 2007; Barbieri et al., 2007; Duda et al., 2011). In this review we analyze the role of CKs in glioblastoma (GBM) development, diffusion and recurrence.

GBM is the most common and most malignant primary glial tumor in adults, characterized by an invariably poor outcome and limited therapeutic options (Dolecek et al., 2012). Standard GBM management involves maximal surgical resection, followed by radiotherapy with concomitant and adjuvant chemotherapy with temozolomide, but in most cases GBM rapidly relapses. Available treatments at relapse are largely ineffective and median overall survival of GBM patients is about 15 months (Stupp et al., 2005).

There is increasing evidence that tumor development, growth, recurrence and resistance to chemo- and radio-therapy is related to the presence of a cell subpopulation, named cancer stem cells (CSCs), nowadays identified in different human hemopoietic and solid cancers, including GBM. Efficient CSC eradication represents the ineludible goal to prevent tumor relapse and thus a target for all new anticancer approaches.

Beside its functional expression in embryonic pluripotent stem cells, in adults CXCL12/CXCR4/R7 axis controls tissue-specific stem cell proliferation (Singh et al., 2013). Similar functions

have been hypothesized to occur also in CSCs. Thus the definition of mechanisms and downstream mediators of CXCR4/R7 activation by CXCL12, in normal and malignant differentiated cells, their progenitors, and in normal and CSCs, is highly relevant for both cancer biology and perspective therapeutic targeting.

CXCL12/CXCR4–CXCR7 SIGNALING

For many years CXCR4 has been considered the unique receptor for CXCL12 and CXCL12 the sole ligand for CXCR4, a singular exception in the CK family that usually shows promiscuous binding within multiple CKs and receptors. Later, CXCR7 (originally named RDC1) was identified as second receptor for CXCL12, showing 10-fold higher affinity for CXCL12 than CXCR4 (Balabanian et al., 2005), CXCR7 is a member of the atypical CK receptor subgroup (ACKR), also including DARC, D6, and CCRL1, that do not activate G-proteins after correct binding with the respective cognate ligand (Bachelerie et al., 2014). ACKR3 has been proposed as the acronym for CXCR7 in this new nomenclature system. Interestingly, CXCL12 shares CXCR7 binding with another CK, CXCL11 (interferon-inducible T-cell α chemoattractant, ITAC) that is also a ligand for CXCR3 (Singh et al., 2013).

In the CXCR7 amino acid sequence, the highly conserved DRYLAIV domain, which controls G-protein binding and activation, is DRYLSIT (Thelen and Thelen, 2008). Typical CK responses, mediated by G protein activity, such as intracellular Ca^{2+} mobilization or modulation of adenylyl cyclase activity, are not generated after CXCR7 binding. Due to the absence of Gi-coupling, CXCR7 was initially proposed to be a decoy receptor, acting as a CXCL12 (and CXCL11) scavenger and able to promote ligand internalization and degradation, to reduce CXCR4 activity (Graham et al., 2012). However, the current vision is that this represents only one of the possible mechanisms by which CXCR7 modulates cellular functions (Sanchez-Martin et al., 2013). Emerging evidence suggests that CXCR7 can activate intracellular signaling pathways, and in particular it is able to elicit Akt, MAP kinase (MAPK), and Janus kinase-signal transducer and activator of transcription (JAK/STAT3) activation, either by direct modulation, through a β-arrestin-dependent pathway (Singh et al., 2013) or after heterodimerization with CXCR4 (Wang et al., 2011; Hattermann and Mentlein, 2013).

CXCL12 is a homeostatic CK, which controls hematopoietic cell trafficking and adhesion, in immune surveillance and development, being constitutively expressed in different organs (e.g., bone marrow, heart, liver, lung, lymph nodes, liver, brain, kidney, pituitary, among others). However, CXCL12 production has been also correlated with pathological processes, such as inflammation, heart failure, cell damage after organ irradiation or during chemotherapy. In particular, CXCL12 secretion is particularly relevant in hypoxic and pro-angiogenic environments within tumors or during autoimmune diseases (Li and Ransohoff, 2009). CXCR4 is also a rather ubiquitous receptor, with a relevant role at the level of endothelial mature and precursor cells and pericytes in healthy conditions and in hypoxic or damaged vascular tissues, including injured carotid arteries and atherosclerotic plaques (Petit et al., 2007).

CXCL12 binding to CXCR4 triggers receptor homo- and heterodimerization, often, but not always, with CXCR7, depending on the co-expression level of receptors (Levoye et al., 2009). Ligand binding changes the CXCR4 three-dimensional conformation favoring heterotrimeric G-proteins GDP/GTP exchange and dissociation into α- and βγ-subunits, that, in turn, activate multiple transductional pathways (Bajetto et al., 2001a): α_i subunits inhibit cAMP formation *via* modulation of adenylyl cyclase activity; the α_q-subunit activates the phospholipase C (PLC)-β, which hydrolyzes PIP2 (phosphatidylinositol 4,5-bisphosphate) inducing the generation of diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3) that controls the release of intracellular Ca^{2+} from ER and the activation of protein kinase C; Gαi subunits also induce the activation of the transcription factor nuclear factor-κB (NF-κB), the Ca^{2+}-dependent tyrosine kinase PYK2, JAK/STAT, and the activation of the phosphoinositide-3 kinase (PI3K)-Akt pathway, leading to cell survival and proliferation. The βγ dimer, acting as a functional subunit, is involved in Ras activation of ERK1/2 MAPK cascade, leading to changes in gene expression and cell cycle progression. CXCR4 also regulates cell survival by the G protein-dependent activation of JNK and p38 MAPKs. Further, βγ dimers interact with ion channels and activate PI3K, modulating CXCL12-dependent chemotaxis. CXCL12 also causes CXCR4 desensitization and uncoupling from G-proteins by GPCR kinase (GRK)-dependent phosphorylation and subsequent interaction of CXCR4 with β-arrestin that mediates internalization of the receptor (Cheng et al., 2000) and targets desensitized CXCR4 to clathrin-coated pits for endocytosis. Moreover, interactions between CXCR4 and β-arrestin also promote the activation of downstream intracellular mediators including MAPKs (p38, ERK1/2) and CXCL12-dependent chemotaxis (Sun et al., 2002). Cell migration is directed by CXCR4 by the formation of a CK gradient controlled by internalization of CXCL11 or CXCL12 bound to CXCR7, without the generation of intracellular signaling (Luker et al., 2009). The formation of CXCR4–CXCR7 heterodimers, modulates CXCR4 signaling (Levoye et al., 2009) and enhances CXCL12-dependent intracellular Ca^{2+} mobilization and ERK1/2 phosphorylation (Sierro et al., 2007), while chemotaxis induced by CXCL12 binding to CXCR4 is blocked by CXCR7 when expressed in the same cells (Decaillot et al., 2011). The enhanced activity of CXCR4–CXCR7 heterodimers in recruiting a β-arrestin complex, provides mechanistic insight into the growth, survival, and migratory advantage provided by CXCR4 and CXCR7 co-expression in cancer cells. β-arrestin recruitment to the CXCR4/CXCR7 complex enhances downstream, β-arrestin-dependent cell signaling (ERK1/2, p38, SAPK/JNK), which induces cell migration in response to CXCL12 (Cheng et al., 2000; Sun et al., 2002; Singh et al., 2013). CXCR7 monomers also promote ERK1/2 phosphorylation and nuclear translocation via G-protein-independent, β-arrestin-mediated signaling (Rajagopal et al., 2010; Decaillot et al., 2011). CXCR7 mediates CXCL12 signaling in cultured cortical astrocytes and Schwann cells that co-express CXCR4. Stimulation of astrocytes with CXCL12 activates ERK1/2, Akt but not p38 which was still evident after gene silencing of CXCR4 but fully abrogated by depletion of CXCR7. Conversely, in Schwann cells CXCL12

triggers also p38 phosphorylation altogether with ERK1/2 and Akt, but these effects require the activation of both receptors (Odemis et al., 2010). A diagram of intracellular transduction pathways related to CXCR4 and CXCR7 activation is depicted in **Figure 1**.

The interaction of CXCR7 with CXCL11 further complicates this chemokinergic system since CXCL11 also binds CXCR3, to induce either proliferative or growth inhibitory signals, depending on the CXCR3 variant (A or B; Singh et al., 2013). Moreover, besides CXCL11, CXCR3 is also bound by CXCL9 and CXCL10 to promote tumor growth, metastasis, angiogenesis and immune cell infiltration into tumors. GBM expression of CXCR3 was confirmed in human and murine GBM cell lines and its activation promotes proliferation *in vitro* and experimental tumor progression *in vivo* (Liu et al., 2011). The biological effects of the above described CK-receptor interactions is strictly related to receptor affinity, crosstalk of shared ligands, and associated intracellular signaling in both normal and tumor cells.

MULTIPLE ROLES OF CXCL12/CXCR4-R7 NETWORK: REGULATION OF EARLY DEVELOPMENT OF THE CNS

CKs are pivotal regulators of cell migration, adhesion, and proliferation not only during inflammation and immune surveillance but also during CNS development. In particular, beside inflammatory or homeostatic leukocyte migration, CXCL12 retains a primordial role, highly conserved through the evolution, in the regulation of embryonic and adult stem cell directional migration. The first evidence of the function of CXCL12 in neural development was suggested by the lethal phenotype of CXCR4- and CXCL12-knockout mice (Ma et al., 1998; Zou et al., 1998), both exhibiting abnormal neuronal migration in the cerebellum, dentate gyrus and dorsal root ganglia, in addition to defective lympho-myelopoiesis, and imperfect vasculature and heart development. During cerebellar development CXCR4-positive granule cell precursors are retained in the external granule layer through their interaction with CXCL12 expressed in the overlying pial meninges, ensuring sufficient cell proliferation and allowing the migration to the internal granule layer only

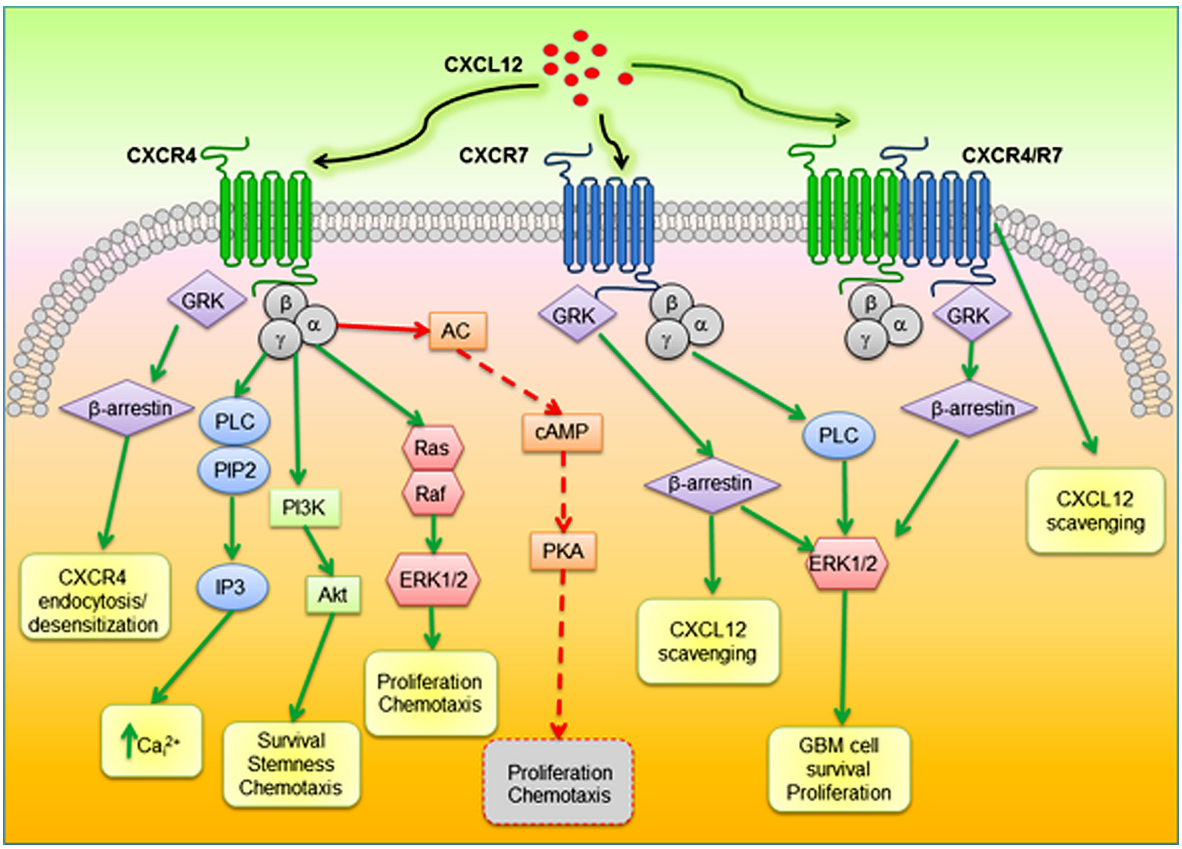

FIGURE 1 | Schematic diagram of proposed CXCR4–CXCR7 crosstalk affecting major signaling pathways related to cell survival, proliferation, and migration. CXCL12 binds to CXCR4 and CXCR7, which can form homodimers or heterodimers. CXCR4–CXCR7 heterodimerization induces a conformational change of CXCR4/G-proteins and blocks signaling. CXCL12–CXCR4 interaction activated by CXCL12 triggers GPCR signaling through PI3K/Akt, PLC/IP3, and ERK1/2 pathways, and mobilization of Ca^{2+} from endoplasmic reticulum *via* inhibition of adenyl cyclase mediated cAMP production, thus regulating cell survival, proliferation, and chemotaxis. Beta-arrestin pathway can be activated through GRK to internalize CXCR4. When CXCR7 binds CXCL12, activation of the β-arrestin may lead to scavenging of CXCL12. In glioblastoma CXCL12/CXCR7 also controls cell survival through ERK1/2. AC, adenylyl cyclase; PLC, phospholipase C; PIP2; phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5 trisphosphate; PI3K, phosphoinositide-3 kinase; ERK1/2, extracellular regulated kinase 1/2; GRK, GPCR kinase

when the cerebellar cortex is ready to receive them. Altered CXCL12/CXCR4 interaction causes a premature migration of granule precursors and disorganized layer formation (Stumm et al., 2003; Huang et al., 2014). CXCL12 anchors cerebellar granule precursors and favors their proliferation synergistically with Sonic hedgehog (Shh). The CXCL12/CXCR4 axis also controls the tangential migration of post-mitotic neurons (Tiveron et al., 2006).

Thus, CXCL12/CXCR4 signaling regulates the development of many structures in the central and peripheral nervous system, including cerebellum, cortex, hippocampus and dorsal root and sympathetic ganglia, controlling migration and positioning of cerebellar granule cells, Cajal–Retzius cells, cortical interneurons, and hindbrain pontine neurons (Li and Ransohoff, 2009; Zhu et al., 2009; Zhu and Murakami, 2012). CXCL12/CXCR4 regulation of stem cell functioning continues also in adults, in neurogenic niches of brain and bone marrow. Hematopoietic progenitors cells (HPCs) are retained in bone marrow through a CXCR4/CXCL12 interaction that regulates also HPCs homing to this niche after transplantation (Kaplan et al., 2007) survival and proliferation. Interestingly, a similar mechanism has been demonstrated in adult neural progenitor cells (NPCs) or neural stem cells (NSCs; Li and Ransohoff, 2009).

First detected in the 1960s (Altman, 1962), two main areas have been identified in adult mammalian brain where NSCs are localized: the subventricular zone (SVZ) along the lateral walls of the lateral ventricles, and the subgranular zone (SGZ) in the dentate gyrus of hippocampus. NSCs have the potential to self-renew, proliferate, and differentiate into neurons, astrocytes, and oligodendrocytes (Laywell et al., 2007), also in response to ischemic or hypoxic insults (Yang and Levison, 2006; Laywell et al., 2007; Miles and Kernie, 2008; Jin-qiao et al., 2009).

In postnatal brain, CXCR4 expression continues in NPCs in SVZ of lateral ventricle and SGZ of dentate gyrus, the adult brain neurogenic areas, while CXCL12 is expressed in ependymal and endothelial cells adjacent to the proliferating areas. CXCL12 triggers the homing of SVZ NSCs from the ependymal niche to the vascular niche that is abrogated by CXCR4 knockdown. Notably, it has been suggested that the migration of responsive cells to the vascular niche, or non-migration of resting cells in the ependymal niche, is regulated by the levels of CXCL12 in the microenvironment (Miller and Gauthier-Fisher, 2009).

Thus, both in adult and embryonic brain, one of the main physiological roles of the CXCL12/CXCR4 axis is the positioning of NPCs near blood vessels or meninges that provide important source of factors for proliferation and differentiation. Generally, CXCR7 has a more limited and less characterized pattern of expression than CXCR4, and it is primarily involved in vascular and cardiac development, rather than hematopoiesis, as observed *in vivo* in CXCR7 knockout mice that die at birth from severe heart and vascular defects (Sierro et al., 2007). However, CXCR7 is also expressed during mouse embryogenesis in the neural tube and brain concomitantly with neural crest development and vascularization. (Schonemeier et al., 2008b). During rat brain development, CXCR7 expression starts at E11.5, increases between E15 and E18 in the marginal zone/layer I, and decreases postnatally. In the cerebral cortex, CXCR7 is expressed in GABAergic neuron progenitors, Cajal–Retzus cells, and neural precursors (Schonemeier et al., 2008a).

The molecular function of CXCR7 within the brain has been investigated by studies in zebrafish that provided the first and strong evidence that CXCR7 acts as scavenger receptor, mediating CXCL12 internalization and providing directional cell migration of primordial germ cells to the gonads and the formation of the posterior lateral line. CXCR4 is expressed by migrating cells and CXCR7 acts by sequestrating CXCL12 from non-target areas, allowing the correct cell migration (Dambly-Chaudiere et al., 2007). In the absence of CXCR7, migrating cells still respond to CXCL12, but their movement ends in undesirable sites because of a specific accumulation that prevents the formation of a CXCL12 gradient required for a directional migration. (Boldajipour et al., 2008; Cubedo et al., 2009). In mammals, the scavenger function of CXCR7 has been established in mouse heart valve, as well as umbilical vein endothelial cells (Naumann et al., 2010) and it was shown to be responsible of interneuron migration. Accordingly, CXCR4 and CXCR7 are co-expressed in migrating interneurons but they have a different subcellular localization: CXCR4 in the plasma membrane and CXCR7 in intracellular recycling endosomes (Wang et al., 2011; Sanchez-Alcaniz et al., 2011). CXCR7 is frequently expressed, in the absence of CXCR4, in forebrain in postnatally generated olfactory interneuron precursors, further demonstrating that CXCR7 is an independent, direct mediator of CXCL12 signaling (Tiveron et al., 2010).

The relevance of CXCL12 and CXCR4/R7, in CNS functions and brain development is even more important considering the consequences of the cancer stem cell hypothesis (Singh et al., 2003, 2004a), based on the concept that tumors derive from cells endowed with stem or stem-like features in which alterations of the self-renewal mechanisms are induced. Therefore, understanding the CK-dependent mechanisms associated with the stemness in normal neural progenitors might help to clarify their activity in cancer development.

ROLE OF CXCL12–CXCR4/R7 IN GLIOBLASTOMA

CXCL12–CXCR4/R7 system plays a central role in tumor development and tumor cell proliferation, mainly acting *via* an autocrine/paracrine mechanism, and contributes to the dissemination and invasiveness of several human cancers, including pancreatic, colon, ovarian, prostate, breast, and renal carcinomas, lymphoma, melanoma, neuroblastoma and GBM (Zlotnik, 2006; Barbieri et al., 2010; Lippitz, 2013; Singh et al., 2013). Moreover, less malignant or benign tumors (i.e., pituitary adenomas, meningiomas, etc.) seem to be regulated by the activity of this chemokinergic axis (Bajetto et al., 2007; Barbieri et al., 2008; Wurth et al., 2011).

Additionally, CXCR4 expression in tumor cells was associated with metastasis of many human malignancies (Muller et al., 2001; Ben-Baruch, 2008; Zlotnik, 2008; Ferrari et al., 2012) favoring their migration and homing toward CXCL12 expressing organs (lung, liver, brain, lymph nodes, bone marrow; Teicher and Fricker, 2010).

The significance of the expression and function of the CXCL12/CXCR4 axis in brain tumors has been intensely investigated in adult and children GBM, astrocytoma, medulloblastoma, oligodendroglioma, and oligodendroastrocytoma (Domanska et al., 2011). In GBM, CXCR4 and CXCL12 are overexpressed in tumor tissue when compared with normal adjacent parenchyma, and their expression level is correlated with tumor grade and poor prognosis (Salmaggi et al., 2005a; Bian et al., 2007). Immunohistochemical studies showed that in GBM CXCR4 and CXCL12 expression does not co-localize with tumor proliferating cells (identified by MIB-1 expressing cells) but they are both mainly localized in hypoxic regions, characterized by necrosis (Rempel et al., 2000; Salmaggi et al., 2005b; Zagzag et al., 2008). In these perinecrotic areas ("pseudopalisading" necrosis), characterized by high cellularity (Rempel et al., 2000; Bajetto et al., 2006; Rong et al., 2006) due to the powerful invasion of glioma cells (Sciume et al., 2010), CXCR4 and CXCL12 co-localize in the same tumor cells (Bajetto et al., 2006). Pseudopalisade areas are peculiar pathologic structures of GBM resulting from a sequence of vascular occlusion and hypoxia (Brat and Van Meir, 2004) leading to migration and accumulation of GBM cells around central necrotic areas and microvascular hyperplasia induced by hypoxic pseudopalisading cells (Jin et al., 2006). Hypoxia promotes GBM angiogenesis, not only *via* hypoxia-inducible factor-1α (HIF-1α) that directly induces the transcription of VEGF and cytokines (i.e., TNF-α), and stimulates CXCL12 expression, but also up-regulates CXCR4 expression in pseudopalisades. Thus, CXCL12 drives angiogenesis either directly or in a paracrine manner, supporting tumor growth and GBM cell migration far from hypoxic pseudopalisades, allowing for both necrotic area formation and peripheral invasiveness of GBM.

On the other hand, CXCR4 and CXCL12 expression frequently occurs in GBM proliferating vascular endothelium, but not in endothelial cells from astrocytomas in which proliferation of microvessels is less abundant (Bajetto et al., 2006).

Interestingly, the mechanism of dissemination of glioma cells within the brain, differently from other cancers, does not occur through lymphatic and hematogeneous spread. GBM cells invade the adjacent brain parenchyma with a morphological pattern known as "Scherer's structures" that include normal brain structures (white matter, blood vessels, and parenchyma) where CXCL12 is highly expressed. GBM cells, organized around neuron and blood vessels in subpial regions and in white matter express high levels of CXCR4: VEGF-dependent CXCL12 up-regulation in neuronal and endothelial cells induces the migration of CXCR4-positive GBM cells, representing the molecular mechanisms of Scherer's structure formation (Ehtesham et al., 2006; Zagzag et al., 2008; Munson et al., 2013). CXCL12 exerts also pro-angiogenic activity, recruiting CXCR4-positive, circulating bone marrow-derived cells (Petit et al., 2007) and promoting tumor vasculature recovery after irradiation, as a consequence of treatment-induced hypoxia and HIF-1α activation, which results in increased CXCL12 expression (Jin et al., 2006; Kioi et al., 2010).

The ability of CXCL12 to guide GBM cell migration has been widely supported by *in vitro* experiments (Rubin et al., 2003; Bajetto et al., 2006) and, although the molecular mechanisms involved are not definitively identified, the effect of CXCL12 results from a functional cooperation with EGFR and PDGFR, overexpressed in GBM cells (Woerner et al., 2005; Sciaccaluga et al., 2013).

The tumor microenvironment, consisting of constitutive non-cancerous cells (fibroblasts, endothelial cells, and immune cells), as well as connective tissue and extracellular matrix, contributes to GBM (and other solid tumors) development, proliferation, invasiveness and angiogenesis (Domanska et al., 2013). CXCL12/CXCR4 axis acts on the tumor microenvironment through the modulation of the expression and secretion of other CKs (i.e., CCL2, CXCL8) and, concomitantly, CXCR4 expression could be influenced by cytokines (TNFα, IFNγ, IL4-6-10) produced by cells in the microenvironment. These interactions represent an indirect mechanism mediating CXCL12/CXCR4-dependent promotion of survival, proliferation and migration of tumor cells (Zhou et al., 2002; Burger and Kipps, 2006).

Hypoxia enhances CXCL12 secretion in cancer-associated fibroblasts which in turn feeds tumor development either by direct stimulation of tumor cells expressing CXCR4 (paracrine effect) or recruiting endothelial cells for angiogenesis (endocrine effect; Burger and Kipps, 2006). Stromal fibroblasts support the growth of neoplastic cells through elevated secretion of CXCL12 (Orimo et al., 2005), and integrins induce expression of CXCR4 and growth-factor receptors sustaining a pro-survival loop for tumor cells.

The interaction of GBM cells with the microenvironment that protects cancer cells from the chemo- and radio-therapy stress, becomes even more relevant in the context of CXCL12/CXCR4 up-regulation observed after treatment with anticancer drugs, and particularly after anti-VEGF antibodies (Shaked et al., 2008; Kioi et al., 2010; Keunen et al., 2011).

Several studies investigated the signal transduction of the CXCL12/CXCR4 axis in normal glial cells or in cell lines derived from human GBMs, being the expression of ligand and receptor almost always reported. CXCR4 and CXCL12 expression was described in primary cultures of rat type I astrocytes, cortical neurons and cerebellar granule cells and treatment with CXCL12 induced proliferation of normal astrocytes through the activation of ERK1/2 and PI-3K pathways (Bajetto et al., 1999a,b, 2001b). In human GBM cell lines (U87-MG, DBTRG-05 and A172), CXCL12 that is released in the extracellular medium, supports cell growth, likely through an autocrine/paracrine mechanism by the activation of intracellular ERK1/2 and Akt pathways (Barbero et al., 2002, 2003). However, differently from normal astrocytes, GBM cell lines show constitutive Akt activation, further increased by CXCL12, and ERK1/2 and Akt are independently involved in cell proliferation. Conversely, the glioma onco-suppressive gene LRRC4 inhibits CXCL12/CXCR4-induced cell proliferation, chemotaxis and invasiveness reducing ERK1/2 and Akt signaling (Wu et al., 2008).

In vivo studies, in which GBM cells are intracerebrally implanted, showed that CXCL12/CXCR4 binding activates matrix metalloproteinases (MMPs) that contribute to the infiltrative

behavior of GBM cells within the brain parenchyma (Zhang et al., 2005).

While CXCL12/CXCR4 activation within both cancer cells and local stroma clearly contributes to GBM cell proliferation, spreading, and survival to therapy, more recent studies demonstrated that CXCR7 is an alternative, or additional, regulator of GBM growth. CXCR7 is up-regulated in all pathological conditions in which CXCL12 activity is enhanced, including neoplastic diseases, and contributes to tumor growth, adhesion, survival, angiogenesis, and invasion of breast, lung and prostate carcinomas (Miao et al., 2007; Wang et al., 2008) and promotes tumor development in mice (Burns et al., 2006). CXCR7 is highly expressed in tumor endothelial, microglial, and GBM cells (Hattermann et al., 2010). CXCR7 controls tumor diffusion through CXCL12 gradients and it is frequently detected in GBM-associated vasculature (Liu et al., 2010). The increase of CXCR7 expression in microvascular endothelial cells during hypoxia (Schutyser et al., 2007; Monnier et al., 2012) favors CXCL12-induced glioma cell migration (Esencay et al., 2013) facilitating the binding of CXCL12 to endothelial cells (Burns et al., 2006; Liu et al., 2010; Dai et al., 2011) and the activation of CXCL12-mediated cell crossing through endothelium (Mazzinghi et al., 2008; Zabel et al., 2009; Dai et al., 2011).

CXCL12, CXCR4–CXCR7 ACTIVITY IN HUMAN GLIOBLASTOMA STEM-LIKE CELLS

In recent years, the CSC theory has gained more experimental validation in addition to the refined theoretical definition. In particular, GBM is the tumor histotype that more precisely matches CSC criteria, in terms of heterogeneity and hierarchical organization of cells, identification of stem cell features in tumor cell subpopulations and, importantly, as far as pharmacological responses. Thus, considering the significant role of CXCL12–CXCR4/R7 axis in normal stem cell biology, it is evident that this chemokinergic system could play a relevant role in GBM CSCs. Moreover, according to genotypic and phenotypic evidence of a more close reproduction *in vitro* of the *in vivo* tumor characteristics of cultures enriched in CSCs, as compared with established cell lines (Lee et al., 2006), recent studies addressed the role of CXCL12 and its receptors in this GBM cell subpopulation (**Figure 2**).

GLIOBLASTOMA CANCER STEM CELLS

Glioblastoma is a complex, heterogeneous tissue characterized by the coexistence of several different cell populations with a hierarchical organization. Among them, a relatively small population exhibits stem-like features, including the capacity to persist in a constant number through asymmetric mitotic cell division (self-renewal capacity), thus representing a drug-resistant cell reservoir to generate differentiated cells (multilineage differentiation potential), that in GBM are represented by differentiated progeny expressing either neuronal or glial markers. After the identification of distinctive stem cell markers, these cells were named cancer stem cells (CSCs). Importantly, besides showing NSC properties, CSCs are tumorigenic, phenocopying the original tumor when xenotransplanted in animal models. For this reason, they are also called tumor-initiating cells (TICs), in order to highlight their tumorigenic potential (Florio and Barbieri, 2012). CSCs have been detected in different hematological and solid tumors (Bonnet and Dick, 1997) and the first evidence supporting the presence of CSCs in GBM was reported in 2003 (Singh et al., 2003; Singh et al., 2004b). Several groups have subsequently described the isolation and characterization of GBM CSCs (Hemmati et al., 2003; Bao et al., 2006a; Liu et al., 2006; Bajetto et al., 2013; Griffero et al., 2009). Initially, the phenotypic characterization of GBM CSCs was based on the recognition of distinctive NSC markers, such as nestin, Sox2, Nanog, Oct4, BMI1, musashi-1, but later on, components of pathways active in brain development were found to be expressed in GBM CSCs, including Notch (Wang et al., 2010a), Wnt (Jin et al., 2011), bone morphogenetic protein (BMP; Piccirillo et al., 2006) and TGF-β (Ikushima et al., 2009). The five-transmembrane domain glycoprotein CD133 (prominin-1), initially reported as one of the most reliable markers to identify GBM CSCs (Beier et al., 2007), although conflicting reports were subsequently published and not labeling all CSC subpopulations, is still considered a key component of CSCs (Grosse-Gehling et al., 2013) acting as a regulator of cell survival inducing PI3K-Akt activation, *via* a direct interaction with p85 (Wei et al., 2013).

Conceivably, the plastic phenotype recently proposed for CSCs, rather than unique cellular marker(s), is the most valid hypothesis, also taking into account GBM cell heterogeneity and the high degree of plasticity that favors its aggressive behavior (Florio and Barbieri, 2012; Tang, 2012; Ruiz-Ontanon et al., 2013; Wurth et al., 2014).

The origin of GBM CSCs is still unclear and controversial. CSCs were proposed to derive from normal NSCs after the accumulation of oncogenic mutations and/or following events mediated by the microenvironment (Calabrese et al., 2007; Hjelmeland et al., 2011). More recently, it was suggested that differentiated neurons or astrocytes can be dedifferentiated and transformed, acquiring CSC-like features to originate histologically different GBMs (Friedmann-Morvinski et al., 2012).

Niches are the specific sites where normal stem cells reside and in adult tissues constitute a spatially distinct microenvironment, which contains stromal cells, blood vessels and high concentrations of extracellular matrix proteins and growth factors. The interaction between stem cells and specific niches is critical for the maintenance of their functional properties, and, in particular, for the balance between self-renewal and differentiation that regulates cell number and tissue homeostasis (Ramasamy et al., 2013).

Like NSCs, GBM CSCs require specific niches in which a permissive environment, ensuring the correct combination of supporting cells (endothelial cells, reactive astrocytes, pericytes, tumor-associated macrophages) and extrinsic factors, is essential for their maintenance and regulation.

There are relevant similarities between NSC and CSC niche:

Vasculature

Blood vessels are an integral component of both neural and cancer stem cell niches. Endothelial cells (EC) modulate NSCs not only by providing oxygen and nutrients, but also through regulation of their capacity to self-renew, proliferate and differentiate (Shen et al., 2004; Charles and Holland, 2010). A comparable and intricate relationship occurs in the niche where CSCs are closely

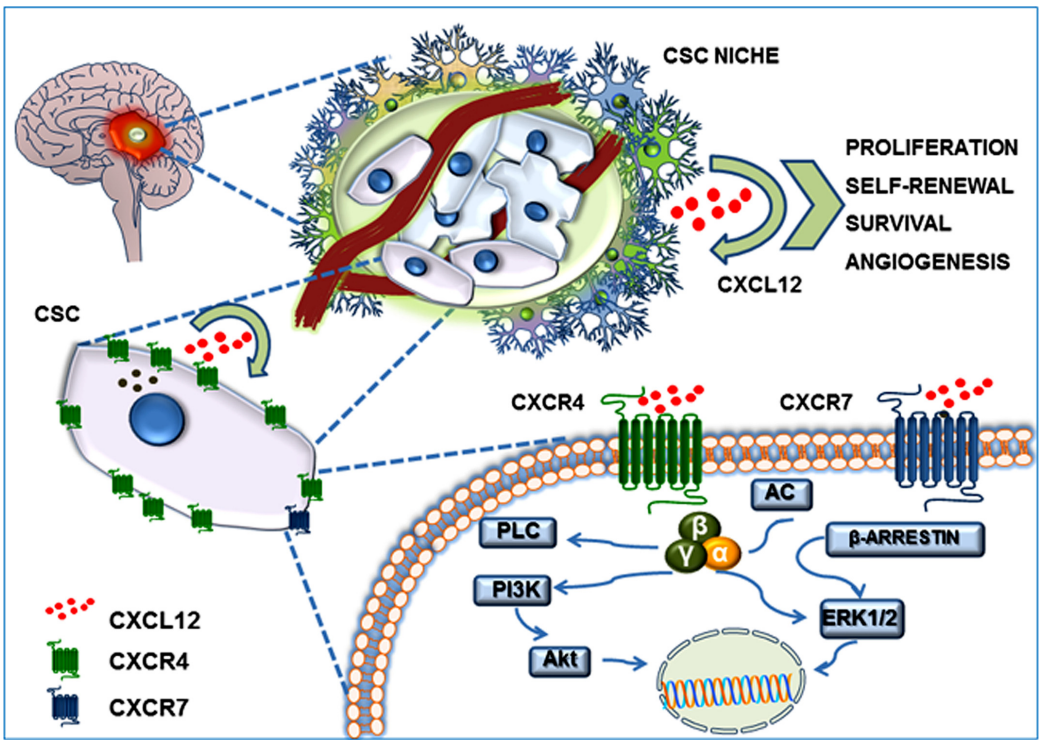

FIGURE 2 | CXCL12/CXCR4–CXCR7 system in the GBM CSC niche. GBM CSC niche is a discrete microenvironment within the tumor mass. It is composed of a heterogeneous cell population that generally includes blood vessels, tumor cells, CSCs, extracellular matrix components, and a gradient of soluble factors. CXCL12 secretion by endothelial cells, tumor cells, and CSCs generates an autocrine and paracrine action which contributes to self-renewal, survival and migration of CSCs themselves, triggering Akt and MAP-kinase(s) intracellular signaling.

connected to ECs establishing a paracrine modulation between these two cell types, largely mediated by the secretion of VEGF and CXCL12 (Calabrese et al., 2007; Yao et al., 2013). GBM CSCs not only release growth factors that induce proliferation of ECs (Bao et al., 2006b) but also may be themselves a direct source of angiogenesis by trans-differentiation into endothelial-like cells. On the other hand, ECs maintain CSCs in undifferentiated state, promote their tumorigenicity (Calabrese et al., 2007) and, *via* the interaction CXCL12–CXCR4, maintain GBM CSCs localized in the perivascular niche (Cheng et al., 2013).

Molecular signaling pathways

Developmental pathways (Notch, Wnt, Shh), and receptors activated by fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor (TGF)- β, and CKs, primarily CXCL12, identified within the niche to maintain NSCs during development and in adult brain, are also involved in CSC survival (Gatti et al., 2013; Penuelas et al., 2009). The interaction between these signal transduction pathways results in self-renewal, sustained proliferation, and increased survival, invasiveness and drug resistance of CSCs (Mimeault et al., 2007).

CXCL12/CXCR4-R7 AXIS IN GBM CSC REGULATION

As previously detailed, CXCL12 modulates tumor cell proliferation, angiogenesis and metastasis, acting as an autocrine/paracrine growth factor (Barbero et al., 2003; Barbieri et al., 2008; Pattarozzi et al., 2008), representing a promising target for the treatment of neoplasia. The concept of CSCs and their identification in several tumors highlights possible new roles for the CXCL12–CXCR4/R7 axis in tumor biology. CXCR4 (over)expression has been detected in CSCs derived from various of cancer histotypes, including pancreatic (Hermann et al., 2007), colon (Zhang et al., 2012), lung (Jung et al., 2013), breast (Dubrovska et al., 2012b) prostate (Dubrovska et al., 2012a), renal (Gassenmaier et al., 2013), and GBM (Singh et al., 2004b). Moreover, the recent demonstration that CXCR7 can also serve as an active receptor for CXCL12 (Odemis et al., 2012) has increased the interest for this chemokinergic system in CSC-related research.

Preclinical studies addressing the role of CXCL12 in GBM CSCs are listed in **Table 1** (generated from PubMed, using "CXCL12–CXCR4–CXCR7-GBM-CSCs" as keywords).

Criteria used to identify and maintain CSC cultures differ among the various studies, mainly because of the absence of absolute and uniform biomarkers and, different methods of CSC isolation and *in vitro* culture enrichment have been reported, making study comparisons difficult. Although putative GBM stem-like cells have been isolated as a subpopulation within established cell lines, the isolation of tumor cell subpopulations from human post-surgical explants, grown as non-adherent neurospheres in serum-free medium enriched with growth factors

Table 1 | CXCL12/CXCR4–CXCR7 axis in GBM CSC, a review of the literature.

CSC isolation	CSC characterization	CSC culture conditions	CXCL12 role	CXCR4–CXCR7 expression	Intracellular pathways	Reference
Primary patient-derived GBM cells	*In vitro* marker expression (nestin), *in vivo* tumorigenicity	Stem cell-permissive medium	Proliferation	High expression of CXCR4 in CSCs		Salmaggi et al. (2006)
Rat glioma cell line (C6)	*In vitro* marker expression (nestin), *in vivo* tumorigenicity	Stem cell-permissive medium (PDGF in place of EGF)	Angiogenesis	$CXCR4^+$ CSCs		Folkins et al. (2009)
Primary patient-derived GBM cells	*In vitro* expression of nestin, CD133, Bmi-1, Sox2, Musashi-1, differentiation potential, and *in vivo* tumorigenicity	Stem cell-permissive medium	Proliferation	Overexpression of CXCR4 in CSCs		Ehtesham et al. (2009)
Primary patient-derived GBM cells	*In vitro* marker exression (CD133), *in vivo* tumorigenicity	Stem cell-permissive medium	*In vivo* proliferation and *in vitro* migration	Overexpression of CXCR4 in CSCs	AMD3100-sensitive CXCL12 activation of Akt and ERK1/2	Schulte et al. (2011)
CD133-cell sorting of primary patient-derived GBM cells and of GBM cell lines	*In vitro* marker expression (nestin, CD133), multilineage differentiation, *in vivo* tumorigenicity	Stem cell-permissive medium	*In vitro* migration; *in vivo* tumor growth and angiogenesis	CXCR4 overexpression in CSCs, whose abolishment diminished *in vivo* tumorigenesis	CXCL12 activated Akt and ERK1/2	Ping et al. (2011)
CD15/CD133 or CD15/L1CAM cell sorting of primary patient-derived GBM cells	*In vitro* marker expression (Sox2 and Olig2), self-renewal, multilineage differentiation, *in vivo* tumorigenicity	Stem cell-permissive medium	*In vitro* and *in vivo* migration and angiogenesis	$CXCR4^+$ CSCs		Cheng et al. (2013)
Primary patient-derived GBM cells	Slow cycling subpopulation	Stem cell-permissive medium	*In vitro* proliferation, migration, self-renewal and angiogenesis	Enrichment of CXCR4 and CXCR7 in the slow-cycling subpopulation		Liu et al. (2013)

(Continued)

Table 1 | Continued

CSC isolation	CSC characterization	CSC culture conditions	CXCL12 role	CXCR4–CXCR7 expression	Intracellular pathways	Reference
Rat GBM cell line (RG2)	*In vitro* marker expression (Nestin, ABCG2, musashi, Oct4, Nanog)	90% DMEM contained 10% fetal bovine serum	*In vitro* and *in vivo* proliferation, *in vitro* self-renewal	$CXCR4^+$ CSCs	Disruption of CXCR4 impaired Akt and ERK1/2 activation	Lee et al. (2013)
Primary patient-derived GBM cells	*In vitro* marker expression (nestin, CD133), multilineage differentiation potential, *in vivo* tumorigenicity	Stem cell-permissive medium	*In vitro* self-renewal, survival, and clonogenicity	Variable expression of CXCR4, minimal expression of CXCR7	CXCL12 activated Akt and ERK1/2	Gatti et al. (2013)
Syngeneic murine CSC line, established from adult mice NSC	*In vivo* tumorigenicity	Stem cell-permissive medium	*In vitro* and *in vivo* proliferation	$CXCR4^+$ CSCs		Uemae et al. (2014)
CD133-cell sorting of primary patient-derived GBM cells	*In vitro* marker expression (CD133), multilineage differentiation potential, *in vivo* tumorigenicity	Stem cell-permissive medium	Self-renewal through CXCR7	Details about receptor expression not provided		Walters et al. (2014)

Stem cell-permissive medium: serum-free medium (DMEM or DMEM-F12 or Neurobasal) with EGF, bFGF and B27, cells growth in non-adherent condition (spheres). When not mentioned, the expression of CXCR7 has not been evaluated.

(Folkins et al., 2009), is currently considered the most reliable source of GBM CSCs (**Table 1**). Long-term passaging of cells in media containing high percentages of serum irreversibly modifies both the phenotype and genotype of the cells as compared to those present in the original tumor, favoring the selection of mutated cells more fit to *in vitro* growth (Lee et al., 2006; Wakimoto et al., 2012). Moreover, early passage GBM CSCs grown under serum free conditions better recapitulate the *in vivo* invasive characteristics of the parental tumor when grown as intracranial xenografts, thus making them a more suitable model system.

The selection of tumor cell subpopulations on the basis of the expression of specific biomarkers common to both NSCs and GBM CSCs (in particular CD133 and nestin), could be performed as an additional step. Culture conditions defined for the propagation of NSCs (serum-free medium containing EGF and bFGF) are effective to sustain GBM CSC growth *in vitro,* allowing cells to grow as floating spheroids (neurospheres) that retain the stem cell phenotype. Noteworthy, neurosphere formation in limiting dilution experiments is one of the main tools used to assess *in vitro* CSC self-renewal (Carra et al., 2013). Finally, CSC isolation has to be further verified by *in vitro/in vivo* analysis of functional properties, such as multilineage differentiation and, most importantly, tumorigenicity in animal models.

The CXCL12–CXCR4 axis was reported as a main regulator of GBM CSC biological features: self-renewal, proliferation, migration, angiogenesis, and chemo-and radio-resistance. Overexpression of CXCR4 was observed in GBM CSCs, which increased proliferation in response to exogenous CXCL12 (Ehtesham et al., 2009). However, this CK is also released by CSCs, suggesting an autocrine/paracrine signaling mechanism (Salmaggi et al., 2006; Gatti et al., 2013). CXCR4 activation in GBM CSCs, in combination with VEGF and HGF signaling pathways under hypoxia, is a key factor in determining NSC tropism toward gliomas (Zhao et al., 2008). Further corroboration of these findings came from studies showing higher CXCR4 expression in GBM CSC cultures than in the differentiated tumor cells obtained from the same culture (Ehtesham et al., 2009). These reports paved the way for further studies that revealed high heterogeneity in CXCR4 expression amongst CSC cultures derived from individual human GBMs (Liu et al., 2013). This observation was supported and highlighted in a recent study showing, in five different CSC cultures, that the distinctive properties of original GBM are retained *in vitro*, including CXCR4 expression and CXCL12 secretion, but were highly variably among cultures, with a general inverse relationship between receptor expression and ligand secretion levels (Gatti et al., 2013).

In vitro, GBM CSC proliferation was induced either by treatment with exogenous CXCL12 (Liu et al., 2013) or by the receptor activation induced by CXCL12 secreted by CSCs in an autocrine fashion (Uemae et al., 2014). This effect was mainly mediated by CXCR4, since it was reversed in the presence of the CXCR4 antagonist AMD3100 (Schulte et al., 2011; Gatti et al., 2013). On the other hand, it was observed that the autocrine effects of CXCL12 promote GBM CSC survival to a higher extent than proliferation: CXCR4 blockade by AMD3100 reduces CSC survival proportionally to the amount of spontaneously released of CXCL12 (Gatti et al., 2013). The ability of AMD3100 to impair colony formation induced by both exogenous and secreted CXCL12 in GBM CD133-positive cells further confirms the autocrine growth-stimulation effect of this CK in this subset of GBM cells (Ping et al., 2011).

The role of CXCL12/CXCR7 axis in GBM CSC biology was only recently investigated and, even if a definitive establishment of its role was not provided, strong evidence supports its involvement in GBM CSC maintenance and tumorigenicity. Pharmacological inhibition of CXCR7 post irradiation caused tumor regression, reduced tumor recurrence, and substantially prolonged survival in a rodent model of GBM, likely interfering with CSCs (Walters et al., 2014). Heterogeneous cell surface expression of CXCR4 and CXCR7, despite similar levels of corresponding mRNAs, was also observed in primary GBM cell cultures. Analysis of cultures enriched in CSCs determined increased percentage of CXCR4- and CXCR7-expressing cells suggesting that both receptors might regulate stem phenotype. Heterogeneous functional responses to CXCL12 are evident, with different roles in promoting *in vitro* cell growth, migration, spherogenesis, and tube formation in individual cultures (Liu et al., 2013). However, $CXCR4^+$, $CXCR7^+$, and $CXCR4^+/CXCR7^+$ cell subpopulations present in cell cultures are all tumorigenic (Lee et al., 2013; Liu et al., 2013).

Conversely, other studies reported that GBM CSCs do not express, or express at a low level, CXCR7 (Hattermann et al., 2010; Gatti et al., 2013). Moreover, upon GBM CSC differentiation, CXCR4 levels diminish while CXCR7 increases, suggesting a prevalent role for CXCR7 in differentiated GBM cells (Hattermann et al., 2010).

The role of CXCL12–CXCR4 axis in GBM CSCs was corroborated by *in vivo* studies. In particular, knocking down CXCR4 using RNAi or inhibiting CXCR4 function by AMD3100 in CSCs, impairs proliferation *in vivo*, effectively reducing tumor growth in two different xenograft models (Ping et al., 2011; Lee et al., 2013); similarly shRNA CXCL12 knock-down in CSCs inhibited tumor growth *in vivo* (Uemae et al., 2014).

SELF-RENEWAL

Besides proliferation and survival, CXCL12/CXCR4 axis plays a significant role in maintaining CSC self-renewal. Self-renewal *in vitro* can be evaluated through sphere formation and clonogenicity assays. These tests were performed in two different models of GBM CSCs (Gatti et al., 2013; Lee et al., 2013) showing that exogenous CXCL12 promoted sphere formation, and that either the pharmacological blockade of CXCR4 by AMD3100 (Gatti et al., 2013) or silencing the receptor (Lee et al., 2013) suppressed CSC sphere-forming ability after serial *in vitro* passages. The role of CXCL12 in CSC sustained self-renewal was also supported by the observation that disrupting CXCR4 signaling reduces the expression of genes associated with self-renewal activity (i.e., Oct4 and Nanog; Lee et al., 2013). Similarly, CXCR7 inhibition by CCX771 powerfully affects CSC self-renewal (Walters et al., 2014). Taken together, these data suggest that maintenance of stemness of the CSC subpopulation represents a relevant function related to CXCL12 activity.

MIGRATION

CXCL12 stimulated *in vitro* migration of CSCs in a dose-dependent manner and co-administration of AMD3100 inhibited peak chemotactic responses. Conversely, the same treatment resulted in minor effects in continuous glioma cell lines, and only in the presence of extremely high concentrations of AMD3100 caused a statistically significant inhibition of migration (Schulte et al., 2011). However, individual CSC cultures displayed heterogeneous responses to CXCL12 in cell migration experiments *in vitro*. In particular, CXCL12 induced AMD3100-sensitive cell migration only in a subset of CSC cultures tested (Liu et al., 2013).

ANGIOGENESIS

CSCs are also responsible for the development of GBM microvasculature. Tumor microvessels were demonstrated to have a neoplastic origin, and CSCs have been suggested to transdifferentiate into functional ECs (Rodriguez et al., 2012). It was demonstrated that GBM CSCs contribute to the microvasculature formation by differentiating into ECs *in vitro* and *in vivo*, and that GBM mouse xenografts contain human-derived ECs (Ricci-Vitiani et al., 2010; Wang et al., 2010b; Soda et al., 2011). More recently, vascular pericytes localized near ECs, have been suggested as the actual tumor-derived cells in neovessels, and, by lineage tracing *in vivo*, GBM CSCs have been proposed to be the source of pericytes rather than ECs (Cheng et al., 2013). CXCL12/CXCR4 axis, at least in part, contributes to GBM pericyte formation inducing migration of CXCR4-expressing CSCs toward the perivascular niche, where ECs secrete CXCL12 (Ehtesham et al., 2009; Folkins et al., 2009) and TGF-β drives differentiation into mature pericytes (Cheng et al., 2013). Furthermore, CXCL12 stimulates VEGF secretion in CXCR4-expressing, $CD133^+$ CSCs from surgical specimens of human GBM and cell lines, promoting tumor angiogenesis via PI3K/AKT signaling (Ping et al., 2011). Discordant findings have been reported using murine GBM stem-like cells, in which endothelial-like differentiation was associated with CXCL12 expression but CXCL12/CXCR4 blockade did not affect either *in vitro* tube formation or *in vivo* angiogenesis. Thus autocrine/paracrine CXCL12 regulates GBM murine stem cell proliferation but probably not angiogenesis (Uemae et al., 2014).

TARGETING CXCL12–CXCR4/CXCR7 AXIS IN CANCER: RATIONALE

The notion that CXCR4/R7 expression in cancer is, in most cases, a negative prognostic factor is well supported (Bian et al., 2007; Maderna et al., 2007). CXCL12/CXCR4 axis is involved in tumor development favoring adaptation, survival and proliferation of cancer cells and CSCs in the tumor environment (Scotton et al., 2002; Zhou et al., 2002; Marchesi et al., 2004) and increasing dissemination of CXCR4-expressing tumor cells in response to CXCL12 gradients (Zlotnik et al., 2011) as CXCL12 is markedly expressed in most common sites of metastasis (liver, brain and bone). Moreover, the pro-angiogenic role of CXCR4/R7 and the ability of CXCL12 to up-regulate and synergize with VEGF support the therapeutic relevance of the pharmacological targeting of this pathway. In particular, CXCL12–CXCR4/R7 axis drives hypoxia-dependent angiogenesis and invasiveness of GBM progenitor cells (Ehtesham et al., 2009). CXCR4 also sustains non-pharmacological resistance of tumor cells, through its effects on the stromal microenvironment that supplies growth- and drug-resistance signals to tumor cells (Burger and Kipps, 2006).

Thus specific targeting of CK receptors or CXCL12 itself in cancer management may provide a valuable tool to modulate autocrine/paracrine signaling networks between cancer cells, CSCs and key stromal components (blood vessels, immune cells, fibroblasts), responsible for tumor cell survival, insufficient drug delivery and reduced efficacy of conventional anticancer drugs. CXCR4/R7-based therapeutics might open up the concept of microenvironment targeted therapy as a new pharmacological strategy, to be used in combination with cytotoxic drugs. Indeed, GBM growth and recurrence relies on CSCs that are responsible for tumor vessel formation and bidirectionally interact with tumor ECs *via* secreted factors, including CXCL12, to preserve stemness and promote self-renewal (Stupp et al., 2005; Calabrese et al., 2007; Gatti et al., 2013).

CXCR4 ANTAGONISTS IN CANCER MANAGEMENT

Most findings on the effects of CXCR4 antagonists in human cancer were obtained in hematological malignancies, disrupting the interactions between CXCR4-expressing leukemia cells and CK secreted by the bone marrow microenvironment. Therefore, results may not be directly translated to solid tumors, but findings could give interesting insights into the potential role of drugs targeting CXCR4, as also shown in tumor animal models.

The discovery of CXCR4 as a co-receptor for T-cell tropic HIV-1 led to the initial development of CXCR4 antagonists such as T140 (Masuda et al., 1992), AMD3100 (De Clercq et al., 1992) and ALX-4C (Doranz et al., 1997). Subsequently, the identification of non-HIV-related functions boosted new applications of CXCR4 inhibitors such as stem cell mobilization, inflammation and cancer treatments.

CXCR4 antagonists can be classified as: (i) modified peptides (T140 and its analogues, BKT140, POL6326, FC131); (ii) small-molecules CXCR4 antagonists (AMD3100, AMD11070, MSX-122, GSK812397); (iii) CXCL12 peptide analogs (CTCE-9908 and CTCE-0214); or (iv) antibodies targeting CXCR4 (MDX-1338/BMS 93656, ALX-0651).

Peptide-based CXCR4 antagonists (TC14012, TZ14001 and TN14003), derived from T140, demonstrated, in preclinical studies, ability to prevent tumor growth and metastasis in animal models of breast, head, and neck carcinoma (Liang et al., 2004; Yoon et al., 2007). In small cell lung cancer cells, TN14003 disrupts CXCR4/CXCL12 interactions and blocks cell adhesion and chemoresistance (Hartmann et al., 2005).

The development of molecules able to inhibit HIV-1/CXCR4 interaction, led to the identification of AMD3100 (De Clercq, 2003), a bicyclam reversible antagonist, the most studied among CXCL12/CXCR4 signaling inhibitors, shown to block CXCL12-mediated calcium mobilization, chemotaxis, and GTP-binding. AMD3100 efficacy in hematopoietic stem cell mobilization was tested in two successful randomized phase III clinical trials on HIV-1 patients (DiPersio et al., 2009) and it was approved by FDA and EMA, in association with G-CSF, for autologous bone marrow

transplantation in multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL)(De Clercq, 2010).

CXCR4 blockade by AMD3100 decreases tumor growth in preclinical GBM models. AMD4365, a novel derivative acting as CXCR4 antagonist inhibits breast tumor formation and reduces lung and liver metastasis (Ling et al., 2013) acting both on tumor and immune cells present in the tumor microenvironment. Association of AMD3100 with bis-chloronitrosourea, showed antitumor efficacy in orthotopic models of GBM demonstrating synergism between CXCR4 inhibition and conventional cytotoxic therapies (Redjal et al., 2006). Currently a combination study with AMD3100 and bevacizumab for patients with recurrent high-grade glioma is ongoing (https://clinicaltrials.gov) with the hypothesis that blockade of CXCR4 will counteract resistance mechanisms to VEGF inhibition. The anti-angiogenic efficacy of AMD3100 was also reported, resulting in a marked reduction of tumor growth and invasiveness in orthotopic GBM-xenotransplanted rats (Ali et al., 2013).

MSX-122, a small molecule identified as a "partial CXCR4 antagonist" (*biased antagonist*) that shows anti-metastatic activity *in vivo* through the unique property of blocking homing and recruitment of cells without mobilizing stem cells (Liang et al., 2012). MSX-122 also inhibits the development of fibrotic process in mice, after radiation-induced lung injury (Shu et al., 2013).

The orally bioavailable derivative AMD11070 powerfully impairs CXCL12/CXCR4-mediated chemotaxis *in vitro* (Mosi et al., 2012; O'Boyle et al., 2013), although phase I/II studies did not warrant further development after safety and pharmacokinetics assessment. BKT140, TG-0054, and POL6326 are currently in clinical evaluation as stem cell mobilizers, for MM, leukemias and lymphomas. The safety and efficacy of BKT140 for mobilization of human $CD34^+$ cells in patients with MM has been recently reported (Peled et al., 2014).

CTCE-9908, a CXCL12 modified peptide, beside hematopoietic tumors, is the only CXCR4 antagonist approved analog by FDA for solid tumors, and specifically for the treatment of osteogenic sarcoma. CTCE-9908 inhibits human breast tumor cells growth in mouse xenografts impairing the CXCR4–VEGF loop and lowering tumor VEGF levels (Hassan et al., 2011) and affects breast, prostate and esophageal cancer metastasization in murine models (Wong and Korz, 2008; Richert et al., 2009). Phase I/II clinical trials of this compound, in patients with hepatocellular carcinoma, are currently under evaluation (Wong and Korz, 2008).

The emerging role of CXCR4 in tumor-stroma cross-talk has a great therapeutic potential to deplete minimal residual disease and CSCs: the disruption of CXCR4-mediated tumor cell adhesion to stromal cells might sensitize residual cancer cells and stem cells to standard cytotoxic drugs. In this respect, MDX-1338/BMS 93656, AMD3100 and BKT140 are currently under investigation in phase I/II clinical studies for MM and chronic lymphoid leukemia (CLL).

The development of antibodies against CK receptors has promising therapeutic efficacy, as reported in preclinical and clinical studies. Pharmacological approaches using antibodies exploit a dual mechanism: direct, by selective functional inhibition of the target receptor and, indirect, by potentiation of host immune response through the recruitment of cytotoxic monocytes/macrophages (i.e., antibody-dependent cell-mediated cytotoxicity) or by binding complement factors (i.e., complement-dependent toxicity). Several CK-directed (CCL2, CCL5, and CXCL10) antibodies have been generated and tested in phase I/II clinical trials (Klarenbeek et al., 2012) in both cancer and inflammatory diseases. 30D8, a humanized antibody against mouse/human CXCL12, inhibits tumor growth and/or metastasis and improve arthritis in experimental *in vitro* and *in vivo* models (Zhong et al., 2013). However, the majority of antibodies that successfully entered clinical trials, targets CK receptors rather than ligands. This development was boosted by the identification of the crystal structure of the receptors, particularly concerning the N-terminal extracellular domain, the most accessible region for antibody binding.

A fully-human CXCR4-targeting moAb, MDX-1338/BMS-93656, able to prevent CXCL12 binding, abolished intracellular Ca^{2+} increase and chemotaxis induced by CXCL12 and it is under study to treat relapsed leukemia or, in combination with lenalidomide/dexamethasone or bortezomib/dexamethasone, relapsed/refractory MM (Kuhne et al., 2013).

A new class of antibody-derived therapeutics, based on single-domain heavy-chain (VHH) antibody fragment, named nanobodies, displays high stability, low toxicity and antigen-binding capacity. The first CK receptor targeted nanobody against CXCR4 (Jahnichen et al., 2010), showed 100-fold higher affinity than AMD3100. CXCR4 nanobodies completely inhibit entry of CXCR4-tropic HIV-1 strains *in vitro* and intravenous injection mobilizes stem cell in animal models, acting similarly to AMD3100 (Jahnichen et al., 2010). Currently, a CXCR4-inhibiting nanobody, ALX-0651, is in phase I trial (https://clinicaltrials.gov: NCT01374503).

Recent insights in structural features of both CXCR4 and CXCL12 and structure-activity relationships, improved chemical modeling and structure-based development of candidate molecules to be screened as CXCR4-antagonists (Wu et al., 2010). A virtual screening of the National Cancer Institute's Open Chemical Repository Collection, using a homology model of CXCR4, led to the identification of a lead structure (Kim et al., 2012). Furthermore, a new family of CXCR4 modulators, as phianidine A, identified by screening a library of marine compounds using a simple pharmacophoric model identified after CXCR4 crystal structure, was recently reported (Vitale et al., 2013).

CXCR7 ANTAGONISTS

In addition to CXCR4, CXCR7 represents a viable target for anticancer and antimetastatic drugs. Inhibition of CXCR7 with selective antagonists in mice engrafted with breast and lung cancer cell lines and experiments testing overexpression or silencing of CXCR7 in tumor cells, collectively support the idea that CXCR7 promotes tumor growth (Miao et al., 2007).

CXCR7 antagonists are expected to act mainly by reducing tumor cell extravasation and thus metastasis, and blocking tumor angiogenesis, as demonstrated by CCX771, a synthetic CXCR7

ligand markedly more potent at inhibiting transendothelial migration than AMD3100. It also stimulates β-arrestin recruitment to CXCR7 in a lymphoblastic leukemia model (Zabel et al., 2009). Moreover, exposure of $CXCR4^+CXCR7^+$ cancer cells to CXCL12 greatly enhances migration of human Burkitt's lymphoma cells through a human HUVEC endothelial cell monolayer as *in vitro* model of transendothelial migration (Zabel et al., 2011) suggesting the potential efficacy of CXCR7 antagonists in blocking CXCL12-mediated metastatic spread of $CXCR4^+CXCR7^+$ tumor cells, *in vivo*.

CXCR7 is also an attractive therapeutic target for hematopoietic stem cell (HSC) mobilization-inducing agents since its expression might be necessary to direct HSCs to the niches sustaining their capacity of migration. Although CXCR7 regulation of BMSC niche has not been completely defined, it should represent a relevant goal for research since the possible inclusion of CXCR7 antagonists in the current formulation of HSC mobilizers (granulocyte-colony-stimulating factor, G-CSF, plus AMD3100) might reduce the percentage of patients in which that mobilization protocol fails (To et al., 2011).

In order to fully elucidate the complex pharmacology and potential therapeutic utility of CXCR7 receptor antagonists, CXCR7 structural models would be highly useful. However, these detailed structures are currently not available. At present, a limited number of CXCR7 ligands have been reported (Kalatskaya et al., 2009; Gravel et al., 2010; Wijtmans et al., 2012), therefore the application of GPCR homology modeling and virtual screening, previously used in CXCR4 studies, for novel CXCR7 ligand identification represents a promising tool (Yoshikawa et al., 2013). AMD3100 and the peptidomimetic CXCR4 antagonist TC14012 have also been reported to act as partial CXCR7 agonists (Kalatskaya et al., 2009; Gravel et al., 2010). Several pharmacological studies with small-molecule CXCR7 antagonists endowed with reasonable affinities have been reported, but none disclosed a structure for the antagonists (Burns et al., 2006; Zabel et al., 2009; Hattermann et al., 2010; Rajagopal et al., 2010; Cruz-Orengo et al., 2011). A recent paper describes the first reported combined synthetic, modeling and pharmacological effort on small molecules targeting CXCR7 (Wijtmans et al., 2012).

DUAL TARGETING OF CXCR4-R7 OR CXCL12 BLOCKADE

Since CXCR4 and CXCR7 are both involved in cancer malignancy, and in particular in GBM angiogenesis, molecules able to interact and block either CXCL12 itself or both receptors simultaneously could represent an improved pharmacological approach (Duda et al., 2011; Singh et al., 2013).

However, as far as dual receptor binding the current available data are rather complex. Some CXCR4 or CXCR7 antagonists were reported to bind also the other receptor although not always acting as antagonist, but partial agonist activity was reported. This is also the case for the prototype CXCR4 antagonist AMD3100 that may act as CXCR7 partial agonist (Kalatskaya et al., 2009). Moreover, CXCR7 agonists selectively activating β-arestin were shown to down-regulate CXCR4 (Uto-Konomi et al., 2013). This differential modulatory effect on the receptors might induce complex biological responses according to the cell analyzed. As far as GBM it was reported that CSCs mainly express CXCR4 while CXCR7 is mainly located in differentiated cells and endothelia (Hattermann et al., 2010; Gatti et al., 2013). In other models the receptors are co-expressed, acting also as heterodimers. Thus, the potential synergism induced by ligands with dual specificity has to be evaluated in the specific cell context and in relation to the agonist/antagonist properties of the molecule.

A more defined picture is obtainable blocking the activity of both CXCR4 and CXCR7 interfering with their ligand. Indeed, synthetic compounds from the family of chalcones, able to bind to CXCL12 with high affinity to prevent its binding to the receptors, have been reported to inhibit inflammatory responses in eosinophils (Hachet-Haas et al., 2008).

Moreover, NOX-A12, an RNA oligonucleotide that binds and neutralizes CXCL12 with high affinity (Liang et al., 2007), is currently in clinical trial for leukemia and MM, displaying antineoplastic activity and stem cell-mobilization from bone marrow. NOX–A12 interferes with CLL cell motility and BMSC-mediated drug resistance, sensitizing CLL cells towards bendamustine and fludarabine, in BMSC co-cultures. Noteworthy, NOX-A12 has been recently reported to be effective in inhibiting or delaying recurrences following irradiation in an *in vivo* GBM model (Liu et al., 2014).

CONCLUSION

In recent years, molecularly targeted drugs have joined conventional chemo- and radio-therapies for the management of several cancers, and have become the first-line treatments for tumors lacking efficacious therapeutic options, such as the approval of bevacizumab for recurrent GBM. Benefits of targeted therapy in terms of overall survival are modest, however in GBM, whose median survival is approximately 15 months, even an improvement of progression-free survival could be encouraging. In this context, the blockade of CXCR4/R7 signaling represents an alternative or additional target for neo-adjuvant treatments. However, a better understanding of the biology of the CK receptors and ligands in CSCs, GBM tissue and stroma, is needed to clarify their role in tumorigenesis and define the actual best therapeutic target among stromal cells, CSC and differentiated cancer cells or their whole cross-talk. In addition, since CXCR4 and CXCR7 are involved in angiogenesis, targeting this chemokinergic system could improve the poor efficacy of inhibitors of angiogenesis in several cancers including GBM.

Conceivably, the combination of CXCR4 and CXCR7 antagonists could represent powerful tool to reduce tumor cell invasion and metastasis. Moreover, the role of CXCL12 pathway in tumor resistance, acting both directly, to promote cancer cell and CSC survival and angiogenesis, and indirectly, to recruit stromal cells that through paracrine activity induce recurrence and metastasis, is crucial for cancer therapy.

However, development of preclinical and translational research targeting microenvironment in hematopoietic and solid malignancies should be paralleled by solution of its limitations as the actual benefit of combination with cytotoxic agents, duration (length) of responses and potential development of mechanisms

of resistance. Moreover, each cancer type might require a different CXCR4 antagonist, exploiting pharmacological features such as oral availability and pharmacokinetics, and the prevalent ability to mobilize hematopoietic cells or to inhibit metastasis or invasion of cancer cells.

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